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CRYSTALLINE BACTERIAL PROTEINASE

VI. PHOSPHOPEPTIDES FROM A BACTERIAL PROTEINASE INHIBITED BY DIISOPROPYL FLUOROPHOSPHATE

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(Received for publication, April 7, 1958)

It is well known that diisopropyl fluorophosphate (DFP) acts as a specific inhibitor for chymotrypsin, trypsin, and many esterases. Phosphopeptides obtained from their diisopropyl phosphoryl-derivatives are known always to contain a phosphorylated serine residue (*1–12*), Serine seems to be one of the important amino acids for the proteolytic and esterolytic activity. The histidine residue is also considered to have an important functional significance (*13–22*), because for example, these enzymes are inactivated as the histidine residue in their proteins is degraded. However, there has been no report that phosphorylated histidine residues have been detected in the hydrolysates of diisopropyl phosphoryl-derivatives of proteinases and esterases.

Previously it has been reported that the proteolytic activity of a bacterial proteinase, BPN′, as well as the esterolytic activity were inhibited by DFP. The derivative of BPN′ was isolated in crystalline form (*23*). Thus, it is natural to investigate the binding site of the phosphorus of DFP and the structure of the peptides adjacent to the active area.

In this paper it will be shown that BPN′ reacts stoichiometrically with DFP and the peptides containing phosphorus are composed of Ser*, Gly, Glu or Glu(NH_2), and some other amino acids.

MATERIALS AND METHODS

BPN′—Twice recrystallized material was prepared as described in the previous report (*25*)** and used after dialysis for 2 days at 5 to 10° against dilute NaCl.

DFP-Inhibited Derivative—DFP-inhibited enzyme was prepared as described previously (*23*) except that the reaction was carried out in 0.1 *M* ammonium-acetate buffer (pH 7.5) instead of phosphate buffer, and used after being recrystallized twice and being dialyzed for 3 days against tap water and dilute buffer solution. The protein concentration was estimated from measurements of the absorption at 280 mμ. It was assumed to be the same as that of BPN′ (*23*), which was also estimated from the dry weight.

Partial Hydrolysis of DFP-Inhibited BPN′ and Separation of Phosphopeptides—The inhibited enzyme (0.5 to 3 g.) was dissolved in 0.1 *M* ammonium-acetate buffer (pH 7.5) and was denatured by heating at 90° for 10 minutes. It was digested with BPN′ (20 to 50 mg.) at 30° for 2 days. Additional hydrolysis was effected by 2 *N* HCl (final concentration) at 105° for 3 to 6 hours in a sealed tube after the precipitate caused by the acid had been removed. This reaction mixture was concentrated, and the HCl removed *in vacuo*. Then the residue was dissolved in a small volume of water and chromatographed on a Dowex-50 column (×8, 200 to 300 mesh, H^+-form, 2×10 cm. for small scale, 2.8×12 cm. for large scale, 4 ml. fractions collected) according to the method of Flavin (*26*). The fractions containing organic phosphorus were collected and treated with dinitrofluorobenzene according to the usual method (*27*). After removing the acidic-ether soluble fraction dinitrophenyl (DNP)-derivatives were extracted with acidic-butanol and chromatographed on an IRC-50 column (150 to 240 mesh, H^+-from, 0.9×30 cm. for small scale, 1.3×32 cm. for large scale, 2 ml. fractions collected) with a solvent (0.3 *N*) HCl : methyl ethyl ketone = 78 : 22 (*v/v*)) used for separation of DNP-amino acids (*28*). The solventes used for paper chromatography were: acidic and basic methanol (*29*), 1.5 *M* ammonium-acetate buffer (pH 5.3 and 6.0), and 0.1 per cent ammonia water-butanol.

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* The abbreviations used for the amino acid residues are those suggested by Brand and Edsall (*24*).

** A commercial lyophilized sample was kindly supplied by Nagase Co., Ltd.

Amino Acid Composition and N-Terminal Amino Acids—Isolated peptides were hydrolyzed for 16 to 24 hours at 105° with 6 *N* HCl. The hydrolysate was treated with dinitrofluorobenzene and analized by chromatography by the usual method (*27*). N-terminal amino acid residues were determined after hydrolysis for 8 to 24 hours.

Detection of Phosphorus—The method of Dryer *et al.* (*30*) was slightly modified using a final concentration of H_2SO_4 of 0.4 to 0.2 *N*, and the final volume was made up to 10 ml. with water.

RESULTS

Phosphorus Content in Crystalline DFP-Inhibited BPN′—Five to 20 mg. of DFP-inhibited BPN′ was digested with H_2SO_4 and the phosphorus content estimated by comparison with a standard phosphate solution. Results are shown in Table I together with those for active BPN′. It was found that as the results of reaction with DFP one mole of BPN′, which originally had

TABLE I

Phosphorus Content of BPN′ and Its DFP-Inhibited Derivative

Sample		Protein concentration* (*mg.*)	Phosphorus concentration (*μg.*)	Mole of phosphorus per mole of protein**
Cryst. BPN′	A	15.3	0	0
	B	5.1	0	0
Cryst. DFP inhibited BPN′	A	16.1	17.0	1.04
	B	8.0	8.7	1.07

* Calculated as BPN′ protein.

** Molecular weight of BPN′ assumed to be 30,500 (*30*).

no phosphorus, contained one mole of phosphorus.

Phosphopeptides from DFP-Inhibited BPN′—Fractions containing organic phosphorus were eluted soon after those containing inorganic phosphorus as shown in Fig. 1. The yield of organic phosphorus was fairly high, varying between 40 and 60 per cent. These fractions were collected, treated with dinitrofluorobenzene, and chromatographed on an IRC-50 column as described above. Further separation and analysis was done using only the main fraction. Fig. 2 shows 2 examples of results of this chromatography. Further separation was carried out by paper chromatography. The procedures used and the results obtained are given below.

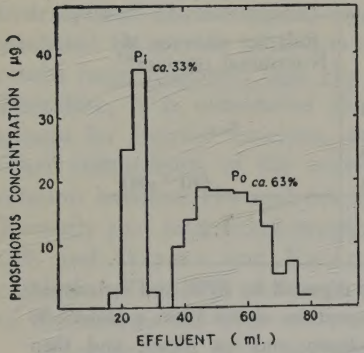

FIG. 1. Elution of phosphorylated peptides from Dowex-50 column.

200 to 300 mesh, H⁺-form, 2 × 10 cm. column. Sample: 830 mg., Partially hydrolyzed by BPN′ for 2 days, and 2 *N* HCl at 105° for 3 hours. Developing solvent: Water first and then 0.05 *N* HCl (40-ml. portions), 4 ml. fractions collected.

P_i: Inorganic phosphorus.

P_o: Organic phosphorus.

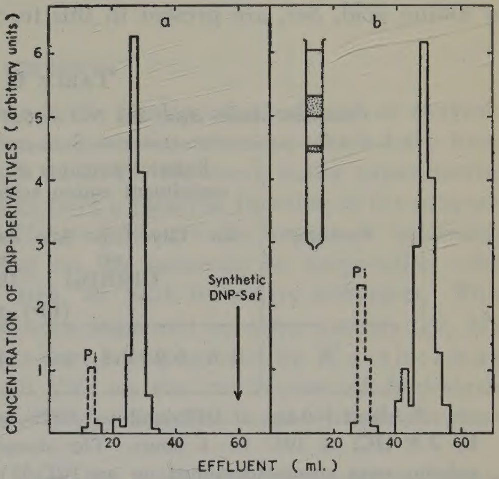

FIG. 2. Elution of DNP-derivatives of phosphorylated peptides from an IRC-50 column.

150 to 240 mesh, H⁺-form, (a) 0.9 × 30 cm. column, (b) 1.2 × 32 cm. column. Sample: (a) 510 mg., (b) 3.1 g. Partially hydrolyzed by BPN′ for 2 days and 2 *N* HCl at 105° for 6 hours (a) and 4 hours (b). Solvent: 0.3 *N* HCl : methyl ethyl ketone = 78 : 22, 2 ml. fractions collected.

P_i: Inorganic phosphorus.

P_o: Organicphosphorus.

The column shows the pattern of the residual DNP-derivatives after elution of main fraction. Synthetic DNP-Ser was eluted in the fraction indicated by the arrow.

Experiment 1—After digestion of 510 mg. of DFP-inhibited BPN′ by BPN′ it was hydrolyzed with acid for 6 hours at 105°. On chromatography one faster moving fraction was obtained in addition to main fraction corresponding to that in Fig. 2-a. When this fraction was chromatographed on paper, only one fraction was in general obtained. The analytical results are shown in Table II. It is shown that Ser is present in higher concentration than other amino acids with a fair amount of Gly and Glu or Glu(NH_2) in the peptides having Ser as the N-terminal residue. Therefore, although this result does not show complete separation of the DNP-derivatives, it seems likely that Ser (Gly, Glu or Glu(NH_2)) and its partially hydrolyzed peptides or amino acid, Ser, are present in this fraction.

TABLE II

Constituent Amino Acids and N-Terminal Residues of Phosphopeptides

Fraction*	Relative amounts of constituent amino acids					Relative amounts of N-terminal residues**
	Ser	Gly	Glu or Glu(NH_2)	Ala	Leu or Ileu (10^{-1} μM)	Ser (10^{-1} μM)
A	1.8	0.8	0.4	++	+	1.2

* About 510 mg. of DFP-inhibited BPN′ was digested by BPN′ and hydrolyzed by 2 *N* HCl at 105° fo 6 hours. The phosphopeptides eluted from a Dowex-50 column were chromatographed on an IRC-50 column and on paper, and then analyzed as described in text.

** No correction made for hydrolytic breakdown of DNP-amino acid.

TABLE III

Constituent Amino Acids and N-Terminal Residues of Phosphopeptids

Fraction*	Organic phosphorus (μM)	Relative amounts of constituent amino acids						Relative amounts of N-terminal residues**
		Ser	Glu or Glu(NH_2)	Gly	Ala	Pro	Leu or Ileu (μM)	Ser (μM)
A	1.3	0.9	0.1	0.03	0.08	0.02	0.1	0.8

* About 3.1 g. of DFP-inhibited BPN′ was digested by BPN′ and hydrolyzed by 1 *N* HCl at 105° for 4 hours. The phosphopeptides eluted from a Dowex-50 column was chromatographed on an IRC-50 column, and then analyzed as described in text.

** No correction was made for hydrolytic breakdown of DNP-amino acids.

Experiment 2—About 3.1 g. of DFP-inhibited BPN′ was analyzed after enzymic digestion and acid hydrolysis. As shown in Fig. 2-b, only one main fraction eluted was directly analyzed and the result was shown in Table III. It was found that almost all the Ser in this fraction was present in the N-terminal residue of the phosphopeptides. There were small amounts of some other contaminating amino acids. The Ser concentration nearly corresponds to that of phosphorus after acid hydrolysis, suggesting that Ser exists as phosphoserine at least in the final stage of analysis. It was also found that after acid hydrolysis the DNP-derivative eluted in the main fraction was eluted in the same fraction as synthetic DNP-Ser from an IRC-50 column.

DISCUSSION

It is known that phosphoserine is the final product of acid or enzymic hydrolysis of chymotrypsin, trypsin, and various esterases, which have been inhibited by specific inhibitors such as DFP. However, many experimental results have suggested that His may have a catalytic function in the enzyme. Therefore, it is considered that His and Ser are the important functional groups for enzyme catalysis, acting on the substrate in cooperation with other components of the active area, or with secondary structures. This relation between His and Ser has been suggested by some authors (*20*, *32*). Recently two interesting hypotheses have been proposed by Westheimer (*33*) and Dixon and Neurath (*34*) on the mechanism of hydrolytic catalysis. The latter authors especially pay attention to the whole structure of the enzyme (*35–37*).

A proteinase, BPN′, obtained from *Bac. subtilis N′* has similar properties to other proteolytic enzymes regarding hydrolysis of proteins and esters (*38*) and behavior towards DFP (*23*), but differs with regard to substrate specificity (*39*) and behavior towards naturally occurring inhibitors (*23*). The results obtained here are similar to those obtained with other hydrolytic enzymes on the site of binding of the phosphorus and the structure of the adjacent peptide. They suggest that the control of specificity may be due to other components than Ser or His in the active area, or to secondary structures in the enzyme.

SUMMARY

The phosphorus content of a bacterial proteinase, BPN′, which had been inhibited by DFP, was analyzed and the site of attachment of the phosphorus, and structure of the adjacent peptide examined. One mole of phosphorus was present per mole of protein. It was shown that Ser must be the amino acid residue binding the phosphorus of DFP. The neighboring peptide contained Gly, Glu or Glu(NH_2), and some other amino acids.

The author wishes to express his thanks to Prof. K. Okunuki and Dr. B. Hagihara for their kind advice during this work. I would like to thank Dr. M. L. Huggins, Research Lab., Eastman Kodak Co., Rochester, N. Y., for a gift of *p*-Semidine, and I am grateful to Nagase Co. Ltd. for kindly supplying the enzyme source materials.

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ADENOSINE TRIPHOSPHATASE OF THE INTRACELLULAR PARTICLES OF ASPERGILLUS ORYZAE*

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(Received for publication, April 15, 1958)

In 1951 Kielley and Kielley (*1*) reported that in rat liver mitochondria prepared in isotonic sucrose there is an increase in adenosine triphosphatase activity (ATP-ase) during incubation which is related to the decrease in oxidative phosphorylation. Since the decrease of oxidative phosphorylation was prevented by addition of adenylic compounds, they assumed that the ageing caused loss of some energy-rich components which are necessary for phosphorylation and thus ATP-ase appeared even in undamaged mitochondria. Furthermore Harman *et al.* (*2*) observed that morphological changes (swelling) of mitochondria of pigeon skeletal muscle are attended by an increase in ATP-ase activity. Mitochondria prepared in isotonic sucrose swell in hypotonic sucrose, and the swelling is promoted by addition of bivalent cations such as magnesium and calcium, and also by uncouplers of oxidative phosphorylation (*3*).

In filamentous fungi, there is little information available on the morphology and physiology of the intracellular particles. This paper presents the results of studies on the ATP-ase of particles from *Aspergillus oryzae.*

EXPERIMENTALS

Aspergillus oryzae (laboratory strain) was cultured on a medium containing per liter of tap water 15 g. of sucrose, 5 g. of KH_2PO_4, 5 g. of $MgSO_4 \cdot 7H_2O$ and trace of $FeCl_3$. The pH was 5.8~6.0. The medium was inoculated with conidial spores and the mycelial mats were harvested after incubation for 40 hours at 30°. They were washed with tap water, blotted dry, and cooled in the refrigerator. One hundred grams of mat was ground in a mortar with 100 g. of sea sand and 100 ml. of ice cold 0.6 *M* sucrose in 0.05 *M* Tris (tris-hydroxy amino methane) buffer at pH 7.0. The mixture was then filtered through gauze. The filtrate was centrifuged at $220 \times g$ for 5 minutes in a Kubota angle head centrifuge. The precipitate was named P 1. The supernatant was centrifuged at $10{,}000 \times g$ for 15 minutes, the second precipitate called P 2. The supernatant fluid was again centrifuged for 15 minutes at $15{,}000 \times g$, and the resulting precipitate named P 3, and the supernatant S. All operations were carried out at $0 \pm 5°$. Fraction P 1 contained nuclei, cell fragments, and uncrushed cells. Fraction P 2 contained spherical particles of 0.5 μ metre diameter which were stained greenish blue by Janus green B aerobically, and changed to reddish purple under anaerobical conditions. Therefore, these were identified as mitochondrial particles. Precipitate P 3 was brownish in color and apparently soluble in sucrose solution.

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* A preliminary report was presented at the Annual Meeting of the Botanical Society at Hiroshima, in October 1955.

Enzymatic dephosphorylation of ATP was measured by the rate of release of inorganic phosphate in a reaction mixture containing 0.3 ml. of 0.01 M Na_2ATP, 0.2 ml. of 0.2 M $MgCl_2$, 0.2 ml. of 2.25 M sucrose, 0.3 ml. of 0.2 M Tris buffer at pH 7.2, and 0.8 ml. of the enzymic preparation. The reaction was started by addition of the enzymic preparation, and stopped after 5 minutes at 30° by addition of 2 M perchloric acid. Appropriate controls were run to correct for the non-enzymic breakdown of ATP, and the release of phosphate, if any, from the enzymic preparation. After low speed centrifugation of the mixture, aliquots of the supernatant were taken for phosphate determination (*4*). The enzyme preparation was diluted with 0.25 M sucrose containing 0.33 M Tris buffer at pH 7.2. Methods for extraction of acid soluble materials, determination of acid-labile phosphorus, pentose, and protein nitrogen, preparation of ATP, and assay of ATP using hexokinase, were the same as described perviously (*5*).

RESULTS

ATP and Acid-labile Phosphorus—The amount of ATP, acid-labile phosphorus, orthophosphate, and pentose in four successive acid extracts of the mycelial mats of *Asp. oryzae* are shown in Table I.

TABLE I

ATP in Successive Acid-soluble Fractions Extracted from the Mycelial Mats of Asp. oryzae

Extraction number	ATP (a)	Acid-labile phosphate (b)	Ortho-phosphate	Pentose (c)	(b)/(c)	(a)/(b)
1	0.15	0.97	1.13	0.27	3.59	0.15
2	0.03	0.66	0.46	0.12	5.50	0.05
3	0.03	0.39	0.16	0.05	7.80	0.08
4	0.01	0.56	0.09	0.02	28.00	0.02
Total	0.22	2.58	1.84	0.46	5.61	0.09

Concentrations of phosphate and pentose are expressed in μ moles per mg. of protein nitrogen. Mycelial mats of *Asp. oryzae* were grown for 48 hours at 30° and extracted with 0.3 N perchloric acid at 0°. 9.5 mg. of protein nitrogen was found per gram wet weight.

A large amount of ATP was found in the first extract, and acid-labile phosphorus compounds other than ATP were found in extracts. It is possible that these were inorganic polyphosphates, because pentose was present in greatest amount only in the first extract. A minute amount of ATP was found in an extract of material which had been homogenized without acid. The ATP may have been destroyed either by ATP-ase or by other enzymic action during the homogenization. Determinations were made of ATP-ase activity and of the acid-labile phosphorus concentration of fractions resuspended in 0.3 *M* sucrose after differential centrifugation (Table II).

TABLE II

Distribution of ATP-ase and Acid-labile Phosphorus in the Cell Fractions

Fraction	Total protein nitrogen (*mg.*)	Total ATP-ase activity*	Specific activity**	Total acid-labile phosphorus (*μ moles*)
P 1	4.48	458	102	7.0
P 2	1.38	225	164	3.2
P 3	1.15	96	84	1.7
S	9.25	4215	455	18.6

* Activity is expressed as μg. phosphorus liberated from ATP in 5 minutes at 30°.

** Specific activity is calculated per mg. of protein nitrogen of the preparation.

P 1, P 2, P 3, and S were prepared as described in the text. 100 g. wet weight of mycelial mat was used.

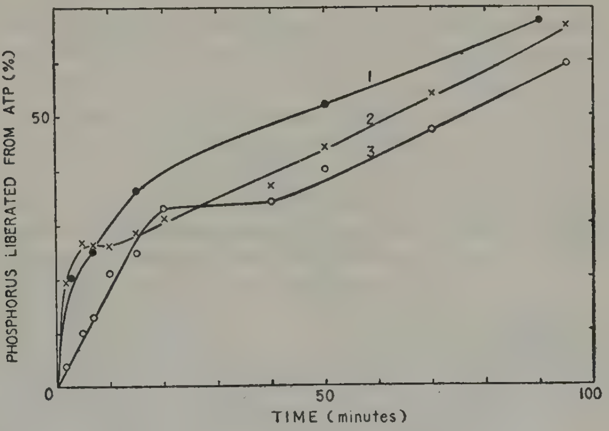


FIG. 1. Apparent ATP-ase activity.

The assay procedure is described in the text. Curve 1: Enzyme extracted with water and precipitated with ammonium sulfate. Curve 2: Fraction S. Curve 3: Fraction P 2.

ATP-ase activity was high in the fraction S and low in the P 3. Table I shows that a large amount of acid-labile phosphorus found in the soluble fraction is not from ATP, but from other inorganic compounds. Thus if active ATP-ase is present in each fraction *in vivo* as shown in Table II, it is unlikely that a large amount of ATP would be detectable in the first extract in Table I. Probable ATP-ase, which is non-functional *in vivo*, owing to inertness or localization, might become active during the homogenization.

Apparent ATP-ase Activity—A water-extract was prepared by homogenizing *Asp. oryzae* mats and was precipitated with ammonium sulfate. This preparation liberated more than one mole of phosphorus per mole of ATP

(Fig. 1). The enzymes in the fraction S and P 2 (mitochondria) gave a distinct two steps reaction; in the first step less than one mole of ATP, and the second more than one mole was dephosphorylated.

The first step is probable due to ATP-ase alone and the second to a combination of adenylate kinase and ATP-ase (*3*, *6*). And also, the dephosphorylation of ATP by the enzyme precipitated with ammonium sulfate showed a two steps curve similar to those in fraction S and P 2. It seems likely that ATP-ase of supernatant is similar in properties to that of mitochondria as described below (see Table V, VI)

Effect on Mitochondrial ATP-ase Activity of Preincubation (*ageing*) *with Isoionic Sucrose*—It is generally accepted that the ATP-ase activity of mitochonaria isolated from animal tissues increases on preincubation as oxidative phosphorylation decreases (*1*, *2*, *7–9*). In the following experiments it was found that the ATP-ase activity of the mitochondrial fraction P 2 increases like that of animal mitochondria during preincubation for 40 minutes at 30° in 0.25 *M* sucrose, but that it does not increase after preincubation at 0° for 60 minutes. Cleland (*10*) has reported that cat heart mithchrondria prepared in isotoic sucrose containing ethylene diamine tetraacetate (EDTA) retain their selective permeability for electrolytes. The EDTA might therefore have a protective action towards ATP from hydrolysis by ATP-ase in ageing mitochondria. However., EDTA was not found to inhibit the increase of ATP-are activity in fraction P 2 during ageing (Table III).

TABLE III

Effect of Ageing and of EDTA on Mitochondrial ATP-ase Activity

Enzyme concentration (relative amount)*	Phosphorus liberated from ATP (*μ moles*)		
	Control (1)	Preincubation	
		without EDTA (2)	with EDTA (3)
100	3.72	5.44	5.65
50	1.72	2.70	3.20
25	0.81	1.34	1.67
12.5	0.45	0.92	1.10

* The value 100 contained 51 μg. of protein nitrogen. (2) and (3) were perincubated for 40 minutes at 30° in 0.05 *M* Tris buffer and 0.25 *M* sucrose. (3) contained in addition 4 μ moles of EDTA. Assay of ATP-ase was carried out as described in the text with 40 μ moles of $MgCl_2$, adding 4 μ moles of EDTA to (1) and (2).

The increase of ATP-ase activity with ageing is specific for structurally intact mitochondria, since no increase with time was found in water extracts of the mycelial mats which had been precipitated with ammonium sulfate.

Harman and Kitayakara (*2*) reported that bivalent cations such as calcium and magnesium stimulated the increase in activity of animal mitochondrial ATP-ase by ageing. Table IV shows that the increase of mitochondial (P 2) ATP-ase activity during preincubation was much greater after addition of magnesium and calcium. Low activity in the presence of calcium is attributed to inhibition of ATP-ase by this cation, though it is activated by magnesium. However, stimulation of the increase in activity by the presence of cations was not found in fraction S or in enzyme preparations after precipitation with ammonium sulfate.

TABLE IV

Effect of Ca^{++} and Mg^{++} on the Increase in Activity of Mitochondrial ATP-ase during Preincubation

Enzyme concentration (relative amount)*	Phosphorus liberated from ATP (μ *moles*)			
	Preincubation with Ca^{++} (1)	Preincubation without Ca^{++}, (Ca^{++} was added in activity test) (2)	Preincubation with Mg^{++} (3)	Preincubation without Mg^{++}, (Mg^{++} was added in activity test) (4)
100	1.94	1.00	9.51	6.72
50	1.60	0.90	5.55	3.97
25	0.82	0.25	2.70	2.10
12.5	0.50	0.10	—	—

* The value 100 contained 129 μg. of protein nitrogen. Enzyme was preincubated for 40 minutes at 30° in 0.05 *M* Tris buffer and 0.25 *M* sucrose. 20 μ moles of $CaCl_2$ was added to (1), and 20 μ moles of $MgCl_2$ to (3). ATP-ase was assayed as described in the text, after addition of 40 μ moles of $MgCl_2$ and 20 μ moles of $CaCl_2$ to both (1) and (2), and with 40 μ moles of $MgCl_2$ to (3) and (4).

Effects of Calcium, Magnesium, and EDTA on the Activity of Soluble ATP-ase—Like the mitochondrial ATP-ase, ATP-ase in water-extracts of the fungi was also activated by magnesium and inhibited by calcium (*6*). Inhibition by calcium was strong at a low, but weak at a high enzyme concentration (Table V).

To recover the activity of preparations inhibited by calcium, the effect of addition of EDTA on the activity of the ATP-ase was examined. At a low enzyme concentration, activity was partially recovered by a addition of EDTA, and inhibited by the high concentration of it. It is assumed that the supression of activity by the EDTA (Exp. No. 7) was due to chelation with magnesium, which activates the enzyme, because inhibition by excess of EDTA was recovered by addition of magnesium (Exp. No. 8).

Release of ATP-ase from Mitochondrial Particles—Fraction P 2 was suspended in 0.2 *M* sucrose (hypotonic) and submitted to successive centrifugation at 0°.

The ATP-ase activity of each supernatant and precipitate was determined (Table VI.)

TABLE V

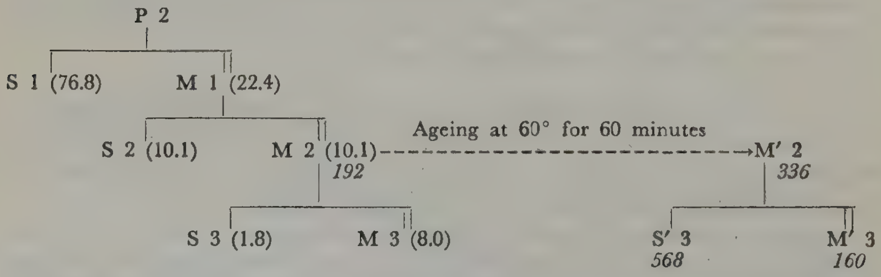
Effect of Ca^{++}, Mg^{++} and EDTA on the Activity of Soluble ATP-ase

No.	Incubation with (μ moles)			Phosphorus liberated from ATP (μ moles)	
	Ca^{++}	Mg^{++}	EDTA	Low concentration of enzyme (30 μg. of protein nitrogen)	High concentration of enzyme (120 μg. of protein nitrogen)
1	—	40	—	6.25	7.95
2	8	40	—	2.09	6.80
3	8	40	2.5	2.72	7.82
4	8	40	5.0	3.55	9.00
5	8	40	7.5	3.70	8.30
6	8	40	10.0	2.30	8.12
7	8	40	15.0	0.68	2.95
8	8	80	15.0	3.32	4.15

Enzyme assay was carried out as described in the text.

TABLE VI

ATP-ase Activity of Mitochondrial Particles Washed with Hypotonic Sucrose



Numerals in the brackets shows the total activity (μ moles of phosphorus liberated from ATP as described in the text) of enzyme corrected to the original mass (100 g. of mycelial mat). Italic numerals shows specific activity (activity per mg. of protein nitrogen). Fraction P 2 suspended in 0.2 M sucrose was centrifuged at 20,000 × g for 30 minutes, and the percipitate (M 1) was resuspended and centrifuged in the same way. The procedure was repeated once more. The supernatants of the first, second, and third centrifugations are denoted as S 1, S 2, S 3 and the respective precipitates as M 1, M 2, M 3, and so on.

As can be seen the activities of S 2 plus M 2 correspond to M 1, and S 3 plus M 3 to M 2. Mitochondrial ATP-ase, therefore, did not increase, but was liberated into the supernatant from the particles during the procedure in which ATP-ase adhering to the surface of mitochondrial particles was removed. Furthermore, solubility of ATP-ase increased by ageing was examined. After preincubation with hypotonic sucrose (0.2 *M*) at 30° for 60 minutes, the ATP-ase of M 2 in Table VI increases from a specific activity of *192* to *336* (M′ 2). However the supernatant and the precipitate, separated from aged mitochondria by the centrifugation as described above, possessed specific activities of *568* (S′ 3) and *160* (M′ 3), respectively. Therefore the mitochondrial ATP-ase which increased on ageing could be liberated from the particles into the medium. After increase in activity by ageing, original amount of the activity still remained in the particles.

DISCUSSION

According to the data of Harman and Kitayakara (*2*), the ATP-ase activity of rat liver mitochondria is increased by cold incubation with hypotonic sucrose (less than 0.2 *M*) accompanying with morphological changes, and this increase is promoted by bivalent cations. In our experiment with mitochondrial particles of *Asp. oryzae* increase in activity at the condition of cool incubation was not observed (Table VI), though thermal ageing increases the activity, but there was marked liberation of enzyme from the particles into the medium. Promoting effect of bivalent cations such as calcium and magnesium on the increase of ATP-ase during preincubation were confirmed with agreement of their data.

Kielley and Kielley (*6*) observed that ATP-ase of disintegrated mitochondria is associated with small particles which are easily precipitated at 110,000×*g* but not at 20,000×*g*. Moreover, Harman and Kitayakara (*2*) reported that ATP-ase was not found in the supernatant of an aged mitochondrial suspension, though there was an increase in protein nitrogen. Contrary to their findings, in the case of fungal mitochondria, much ATP-ase was liberated from the particles into the medium during ageing.

It has been suggested that uncoupling of oxidative phosphorylation of mitochondria causes some modification of transphosphorylase, and so that ATP-ase activity of mitochondrial particles appears (Hunter (*8*)). Structual changes (swelling) of the mitochondria may proceed the enzymic changes (*e.g.* increase of ATP-ase) (*3*).

The present data on the intracellular distribution of ATP-ase (Table II) show that a large amount of ATP-ase is found in the soluble fraction. However, ATP is undoubtedly present in mycelial mats (Table I, and Reference (*11*)). Thus, ATP-ase in the supernatant fraction may be an artifact due to preparation procedures. This possibility is supported by the fact that the soluble ATP-ase of *Asp. oryzae* behaved in the same way as mitochondrial ATP-ase towards magnesium and calcium.

SUMMARY

1. Mitochondrial fractions of *Aspergillus oryzae* prepared in sucrose possess ATP-ase activity and the activity increased during incubation at 30°. This

increase was stimulated by addition of magesium and calcium.

2. Apparent ATP-ase activity of the mitochondrial fraction showed a two steps curve, the second probably resulting from the combined action of adenylate kinase and ATP-ase.

3. ATP-ase of the soluble fraction is also activated by magnesium, inhibited by calcium, and gives a two steps curve like the mitochondrial fraction.

4. During ageing of the mitochondrial fraction, ATP-ase is liberated from the particles into the medium.

The authors wish to express their gratitude to the Ministry of Education for a Grant in Aid for Fundamental Scientific Research.

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STUDIES ON CYTOCHROME C

III. DETERMINATION OF "NATIVE" MAMMALIAN HEART MUSCLE CYTOCHROME C AND ITS PHYSIOLOGICAL PROPERTIES

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Bodo first succeeded in the crystallization of cytochrome c from the king penguin (*1*). We have duplicated his work by crystallizations of cytochrome c's from the more convenient mammalian sources; horse, bovine and pig heart muscle (*2*, *3*). We have also crystallized cytochrome c from a microbial source; baker's yeast (*2*, *4*). During purification from baker's yeast, cytochrome c in its oxidized form could be separated into several different fractions by chromatography on an Amberlite XE-64 column (*2*). These fractions had almost the same absorption spectrum in their oxidized and dithionite reduced forms and were reduced enzymatically at almost the same rate by lactate in the presence of yeast lactic dehydrogenase. They were also oxidized at the same rate in the presence of "Green brei" (*2*, *5*). These facts obscured which fraction on the chromatogram was identical with the normal cytochrome c in the respiring yeast cells (native cytochrome c). Therefore, after comparison of the chromatographic patterns of cytochrome c extracted and purified by various drastic and mild procedures, one fraction of a possibly native form among the various chromatographic fractions was examined. The yeast cytochrome c crystallized from this fraction was more resistant to the digestions by trypsin and by bacterial proteinase than those of the other fractions. This is in accordance with an idea that a globular protein may be more resistant to the digestion by proteinases than one in which the secondary structure of the protein has been somewhat injured. However, native yeast cytochrome c was found to be digested much more rapidly in its oxidized than in its reduced form (*2*, *5*, *6*).

During the above investigations, cytochrome c's crystallized from the mammalian sources were found to be much more resistant to the proteinase-digestion than crystalline yeast cytochrome c, though having the same absorption spectrum as that from yeast. This paper is concerned with a critical investgation of methods for purification of mammalian cytochrome c's, and with studies on some of their properties with the object of identifying native mammalian cytochrome c's.

MATERIALS AND METHODS

Purification and Crystallization of Cytochrome c's from Bovine, Horse, and Pig Heart Muscle—This was carried out fundamentally according to Bodo's method (*1*) and our method described in the previous paper (*2*, *3*). In the following paper, these cytochromes from the mammalian heart muscles are referred to as bovine, horse or pig cytochrome c.

Preparations of Oxidized and Reduced Cytochrome c—Cytochrome c in phosphate buffer was completely oxidized and reduced by the addition of a minimum amount of potassium ferricyanide and sodium dithionite, respectively. The resulting solutions were dialyzed for one day in a refrigerator against several changes of phosphate buffer. The dialyzed samples contained a small amount of the cytochrome c in an undesired form, especially in the case of reduced samples. Therefore, the dialyzed cytochrome c that had been oxidized (or reduced) was called " oxidized (or reduced) cytochrome c ", to distinguish it from ferri- (ferro-) cytochrome c.

Digestion of Cytochrome c by Bacterial Proteinase and Trypsin—Digestion was carried out as described in our previous papers (*2*, *5*).

Chromatography of Cytochrome c on a Cation Exchange Resin—Amberlite XE-64 (250-300 mesh) was activated by the method described for yeast cytochrome c (*2*) and then buffered to pH 7.0 with $(NH_4)_2HPO_4$-H_3PO_4 buffer of pH 7.0 (0.25 *M* with respect to the ammonium ion). Cytochrome c was chromatographed in its ferricyanide-oxidized form using the same buffer as developer. On the chromatogram, mammalian cytochrome c's separated into two fractions, one of which was easily developed and the other not. Both these cytochrome c's showed the same chromatographic behaviours on re-chromatography, respectively, unless they were drastically treated.

Purification of Yeast Lactic Dehydrogenase—This enzyme was prepared from baker's yeast as previously described (*7*). Its contains cytochrome b_2 and a flavin and can reduce yeast cytochrome c directly without addition of a co-enzyme.

Determination of the Biological Activity of Cytochrome c—The biological activity of cytochrome c was measured by reduction by lactate in the presence of yeast lactic dehydrogenase. The reaction mixture was as follows; 1.0 ml. of 0.05 *M* sodium lactate at pH 6.0, 2.0 ml. of oxidized cytochrome c in 0.2 *M* phosphate buffer at pH 6.0 and 0.5 ml. of yeast lactic dehydrogenase. The reduction of cytochrome c was initiated by addition of enzyme and followed by measuring the increase in optical density at 550 mμ in a Shimadzu photoelectric spectrophotometer, type QB-50.

Measurement of Autoxidizability of Cytochrome c—Autoxidation of ferrocytochrome c was measured with a Warburg manometer at 37°, and ascorbate was added to reduce the ferricytochrome c as its continuous reductant. The reaction mixture was as follows; 1.0 ml. of 0.05 per cent cytochrome c (calculated on the assumption that its molecular weight was 13,000), 0.5 ml. of 0.2 *M* phosphate buffer at pH 7.2, 0.1 ml. of 0.1 *M* disodium ethylenediaminetetracetate and 0.2 ml. of 5.0 per cent sodium L-ascorbate at pH 7.2. The reaction was started by adding the ascorbate, and the oxygen consumption was measured. For measurement of the autoxidation of ascorbate, water was used in place of the cytochrome c. Values for autoxidation of cytochrome c are given after correction for this blank reading.

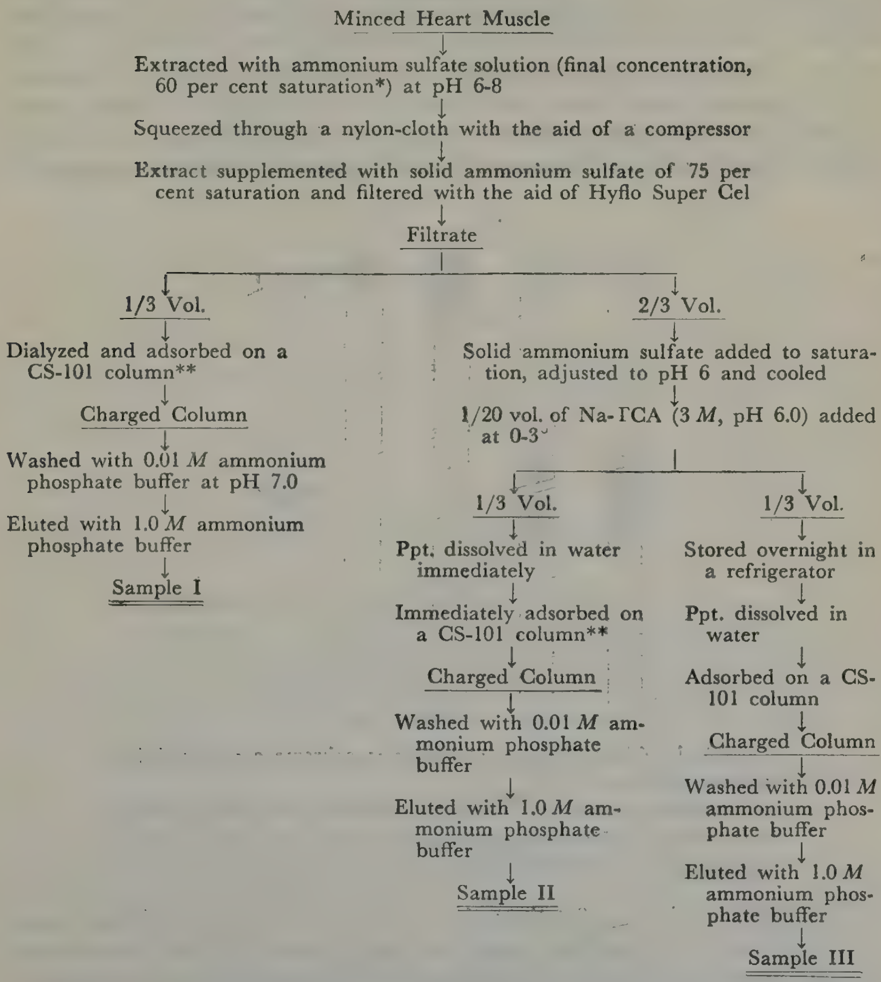
Measurement of the Absorption Spectra of Cytochrome c at pH 7 and at pH 12—2.5 ml. of 0.25 *M* phosphate buffer at pH 7 or 12 was added to 0.5 ml. of the cytochrome c solution which had been dialyzed against 0.01 *M* phosphate buffer at pH 7.6. The absorption spectrum was measured immediately at room temperature from 230 to 320 mμ with a Beckman ratio recording spectrophotometer. The buffer of pH 12 was prepared from

0.5 M Na_2HPO_4 and 0.5 M NaOH.

RESULTS

Effect of Extraction- and Purification-Methods for Cytochrome c from Bovine Heart Muscle on Its Chromatographic Species—Based on the purification procedures described previously (*3*), three types of method were used for purification of cytochrome c from bovine heart muscle. All operations were made in a

DIAGRAM 1


Minced Heart Muscle
Extracted with ammonium sulfate solution (final concentration, 60 per cent saturation*) at pH 6-8
Squeezed through a nylon-cloth with the aid of a compressor
Extract supplemented with solid ammonium sulfate of 75 per cent saturation and filtered with the aid of Hyflo Super Cel
Filtrate
1/3 Vol.
Dialyzed and adsorbed on a CS-101 column**
Charged Column
Washed with 0.01 M ammonium phosphate buffer at pH 7.0
Eluted with 1.0 M ammonium phosphate buffer
Sample I
2/3 Vol.
Solid ammonium sulfate added to saturation, adjusted to pH 6 and cooled
1/20 vol. of Na-TCA (3 M, pH 6.0) added at 0-3°
1/3 Vol.
Ppt. dissolved in water immediately
Immediately adsorbed on a CS-101 column**
Charged Column
Washed with 0.01 M ammonium phosphate buffer
Eluted with 1.0 M ammonium phosphate buffer
Sample II
1/3 Vol.
Stored overnight in a refrigerator
Ppt. dissolved in water
Adsorbed on a CS-101 column
Charged Column
Washed with 0.01 M ammonium phosphate buffer
Eluted with 1.0 M ammonium phosphate buffer
Sample III

* Calculated assuming that water-content of the muscle is two thirds of the total.

** Duolite CS-101 (150–200 mesh) was buffered by the method used for Amberlite XE-64 and washed with distilled water. This resin appears to have a slightly higher capacity to adsorb cytochrome c than Amberlite IRC-50.

pH range between 6.0 and 8.0 at below 5°. As shown in Diagram 1, cytochrome c was extracted from the minced bovine heart muscle by 60 per cent saturated ammonium sulfate to prevent catalytic action of proteolytic enzymes present in the muscle, although cytochrome c could be extracted maximally with a 5–10 per cent solution of the salt and to a lesser extent in its absence. Of these three procedures, sample I was that prepared by what was presumably the mildest procedure. Samples II and III were obtained by the procedures which entailed treatment with neutralized trichloroacetic acid (TCA) for a short and a long time, respectively. Cytochrome c from yeast had been found to be very sensitive to this treatment (*2*). When these samples were chromatographed on an XE-64 column, bovine cytochrome c in its oxidized form was separated into fractions, 1 and 2. As shown in Fig. 1, sample I contained only fraction 2, while the samples II and III separated into fractions 1 and 2. Extraction by 10 per cent saturated ammonium sulfate gave the same results. When eluted with 0.25 *M* ammonium phosphate buffer of pH 7.0, fraction 2 developed typically, while fraction 1 did not. Fraction 1 was eluted easily with 1 *M* buffer, and it is possible that like yeast cytochrome c (*2*) it could have been separated into various fractions by a suitable concentration of the buffer. The cytochrome c's of fractions 1 and 2 gave scarcely distinguishable spectra both in their oxidized as well as their dithionite-reduced form. When fraction 2 eluted with 0.25 *M* buffer was heated for twenty minutes in a boiling

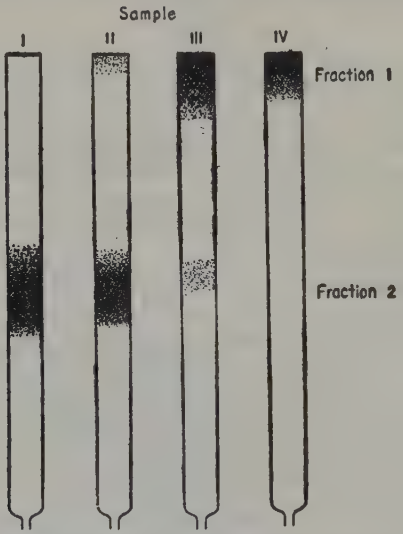


FIG. 1. Chromatograms of bovine cytochrome c prepared by various procedures.

(I), (II) and (III) correspond to Samples I, II and III, respectively, in Diagram 1. (IV) was from Sample IV which prepared by heating Sample I in a boiling water-bath for twenty minutes.

water bath, the resulting sample (sample IV) had almost the same absorption spectrum as before and on the chromatogram developed as fraction 1. On re-chromatography fractions 1 and 2 developed as previously.

Digestion of Bovine Cytochrome c by Bacterial Proteinase—As described in our previous papers (*2*, *5*, *6*), baker's yeast cytochrome c in its oxidized form is easily digested by bacterial proteinase and by trypsin, while it is hardly digested in its reduced form. Bovine cytochrome c also was digested easily by these proteinases as shown in Fig. 2, though its susceptibility to digestion was much less than that of yeast cytochrome c. Samples I, II and III contained more than 95 per cent of cytochrome c even at this stage of purification yet these fractions were digested by bacterial proteinase at very different rates. These were in the following order; sample I≤sample II≪ sample III≪sample IV. Bovine cytochrome c was crystallized from fraction 2. However, it was difficult to crystallize it from fraction 1.

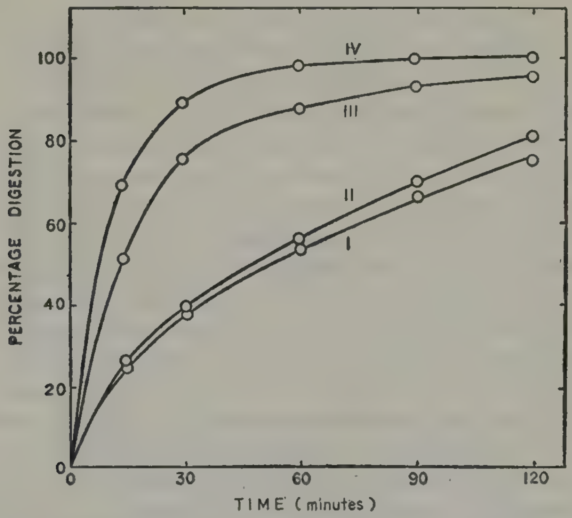


Fig. 2. Digestion of bovine cytochrome c by bacterial proteinase.

The reaction was carried out at 30° under air. The reaction mixture was as follows; oxidized cytochrome c 0.07 per cent and bacterial proteinase 0.017 per cent, in 0.05 M of phosphate buffer of pH 7.2. (I), (II), (III) and (IV) correspond to Sample I, II, III and IV, respectively, in Diagram 1 and Fig. 1.

Similar results were obtained using a trypsin solution which had been adjusted to digest casein at the same rate as the bacterial proteinase solution.

Crystallization of Bovine Cytochrome c—After dialysis in a refrigerator against 0.001 M NaCl or ammonium phosphate buffer of pH 7.0, fraction 2 from an XE-64 chromatogram was re-charged on to a column packed with a minimal amount of the same resin which had been buffered at pH 7.0 and was then washed with distilled water. The charged column was slowly eluted with

10 per cent saturated ammonium sulfate (pH 8). Solid ammonium sulfate was added at pH 8 to the resulting concentrated cytochrome c solution to approximately 30 per cent saturation. After careful adjustment to approximately pH 8 with 5 per cent ammonia, the solution was reduced with a minimum quantity of sodium dithionite or sodium ascorbate. The former reductant was more useful than the latter, for it could be easily removed by aeration and gave more complete reduction. It was possible to estimate the completeness of the reduction by the colour change of the solution from dark to light red with a definite increase in transparence. While maintaining the pH at approximately 8 with ammonia solution, the reduced cytochrome c solution was supplemented gradually with finely powdered ammonium sulfate, till it became slightly turbid. Turbidity then gradually increased spontaneously, and crystals of bovine cytochrome c were formed after 2 to 10 hours. The solution was kept at about pH 8 by using a stoppered tube to prevent vaporization of the ammonia, for cytochrome c was strongly autoxidizable at a lower pH. Too rapid increase in turbidity resulted in the formation of an amorphous precipitate of the cytochrome c. However crystals could be obtained either if this solution was kept for a few days, or by adding a few drops of water untill the solution became slightly turbid again, readjusting the pH and repeating the reduction. At a high concentration of ammonium sulfate, and at the pH cytochrome c was definitely more viscous in its oxidized than in its reduced form, and was less soluble in its reduced than in its oxidized form. For crystallization it was best to start with the cytochrome c solution of an optical density at 275 mμ of between 20 and 50. Single and clumped needle-like crystals appeared (*3*); the crystals formed in a regular manner as rosettes or sheaves appeared in solutions of bovine, horse and pig cytochrome c, but these have not yet appeared in a solution of yeast cytochrome c. The crystalline form of these three mammalian cytochrome c's were more similar to each other than to that of yeast cytochrome c. Crystals of yeast cytochrome c were initially long slender needles and these then grew larger and longer giving rectangular or hexagonal shapes. The crystals were washed three times with 90 per cent saturated ammonium sulfate at pH 8 and were then dissolved in a small amount of phosphate buffer of pH 8 or the distilled water previously adjusted to pH 8 with ammonia. The solution was almost entirely in the reduced form and could be re-crystallized within a few hours without addition of reductant by addition of solid ammonium sulfate while maintaining the solution at pH 8. This re-crystallized cytochrome c had the same chromatographic behaviours as the initial fraction 2.

Digestion of Crystalline Bovine Cytochrome c in Its Oxidized and Reduced Form by Bacterial Proteinase—As shown in Fig. 3, reduced bovine cytochrome c was much less susceptible to digestion by bacterial proteinase than the oxidized form. The time-course of the digestion of the reduced cytochrome c in aerobic conditions followed a complicated curve with a point of inflection. However, when treated with proteinase anaerobically, only a little co-existing ferricytochrome c in the reduced sample was digested. Therefore, probably under aerobic conditions, the ferricytochrome c contaminating the reduced sample was digested first and then this catalyzed the oxidation of ferro- to ferricytochrome c which was susceptible to the proteinase-digestion. This explanation seems likely, since in the case of yeast cytochrome c partially digested ferricytochrome c has been found to catalyze the oxidation (*5*). Under the present conditions, autoxidizability of reduced bovine cytochrome c was negligible unless proteinase was present.

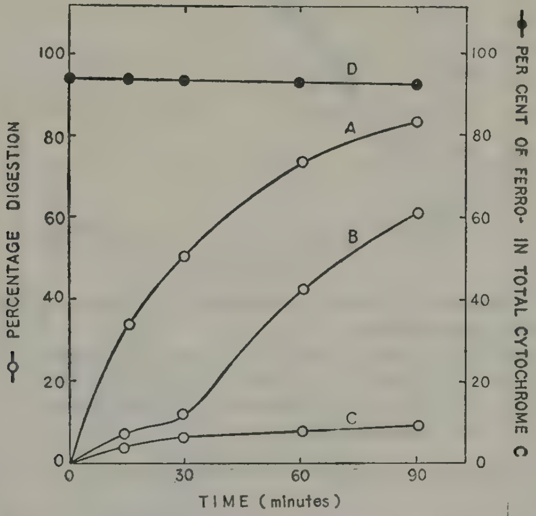


FIG. 3. Comparison of digestion of oxidized and reduced crystalline bovine cytochrome c by bacterial proteinase.

Experimental conditions as in Fig. 2 except for the following: Reactions (A), (B) and (D) were carried out under air, and (C) under nitrogen. (A), (B) and (C) show "percentage digestion", and (D) shows "percent of ferrocytochrome c in total cytochrome c". (A), oxidized crystalline bovine cytochrome c; (B) and (C), reduced form (94 per cent of ferrocytochrome c); (D), reduced form in the absence of bacterial proteinase.

Using trypsin (having almost the same activity as bacterial proteinase towards casein) similar results were obtained.

Effect of TCA-Treatment and Boiling of Crystalline Bovine Cytochrome c on Its Reduction by Yeast Lactic Dehydrogenase—When crystalline bovine cytochrome c was treated with TCA at a neutral pH for a long time or was boiled, its chromatographic species and susceptibility to proteinase-digestion changed in the same way as occurred during the purification procedures. However, neither the ability to be reduced by yeast lactic dehydrogenase (Fig. 4) nor the absorption spectra of the oxidized and dithionite-reduced form were affected.

Effect of TCA- Treatment and Boiling of Bovine Cytochrome c on Its Autoxidizability—It is well known that after reduction by ascorbate cytochrome c

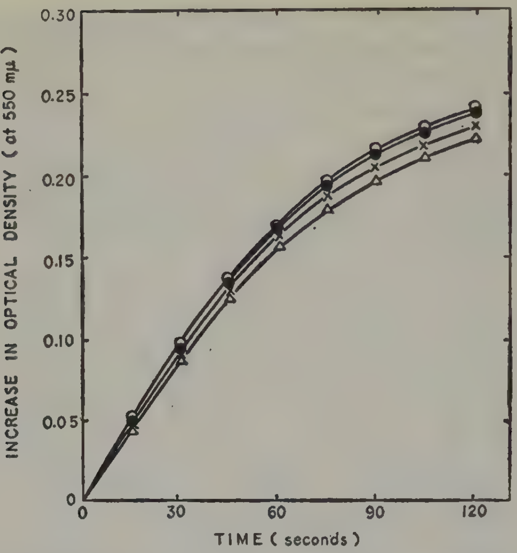


FIG. 4. Reduction of bovine cytochrome c by lactate in the presence of yeast lactic dehydrogenase.

The reaction was carried out at 15° using cytochrome c at a final concentration of 0.025 per cent. Samples I, II, III and IV were those of Diagram 1 and Fig. 1. —○—, Sample I; —●—, Sample II; —×—, Sample III; —△—, Sample IV.

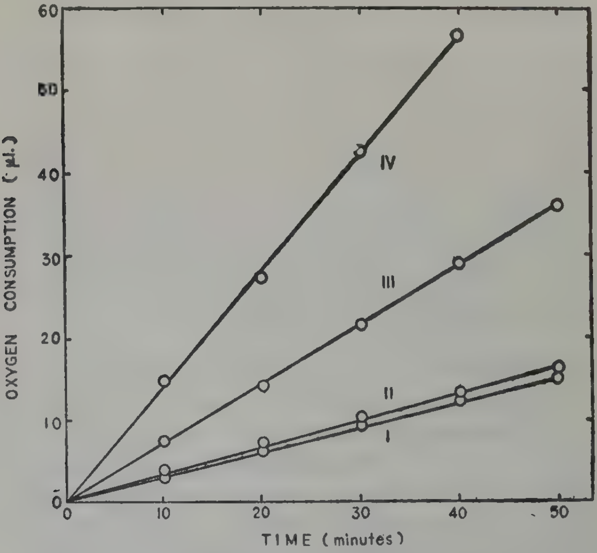


FIG. 5. Autoxidizability of bovine cytochrome c.

(I), (II), (III) and (IV) correspond to Samples I, II, III and IV in Diagram 1 and Fig. 1. Crystalline bovine cytochrome c and its boiled form gave the same results as (I) and (IV), respectively.

can be autoxidized under a strictly aerobic condition. As shown in Fig. 5, the autoxidizability of the four samples obtained by the various purification procedures were in the same order as for the proteinase-digestion, that is; sample I≤sample II≪sample III≪sample IV. The crystalline bovine cytochrome c had the same autoxidizability as sample I and when boiled it was like sample IV. This shows that the more drastic the treatment of crystalline bovine cytochrome c, the higher is the autoxidizability. It shows also that fraction 2 of the resin chromatogram and crystalline cytochrome c were the most native forms.

Ultraviolet Absorption Spectrum of Crystalline Bovine Cytochrome c in Its Oxidized and Reduced Form—Ungar *et al.* (*8*) have expressed some changes of protein-configuration by the ratios of the extinction of the protein at pH 12.0 to that at pH 7.0, terming the ratio as "side-group ionization ratio" (SGIR).

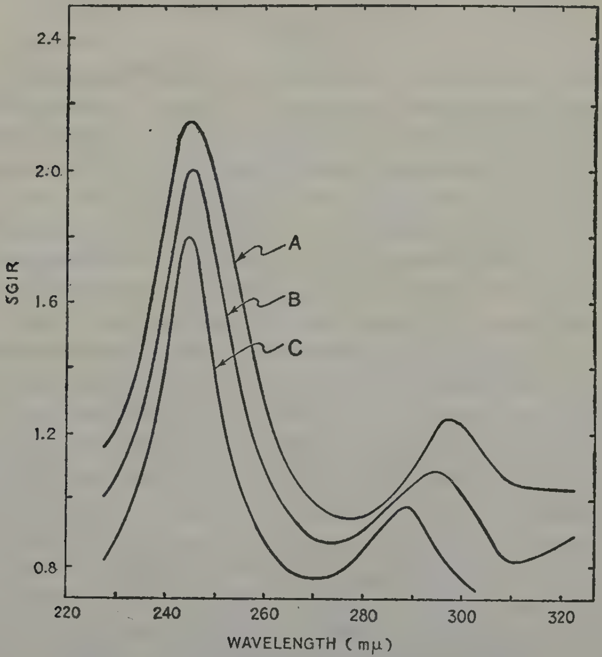


FIG. 6. SGIR for oxidized and reduced crystalline bovine cytochrome c. (A), oxidized crystalline bovine cytochrome c (with negligible contamination by ferrocytochrome c); (B), ferro-/ferri-cytochrome c=4/6; (C), reduced cytochrome c (97 per cent ferrocytochrome c).

They have found that if SGIR are plotted against the wave-length, a protein shows two peaks around 245 mμ and 300 mμ and that these two peaks of a denatured protein are higher than those of the native form. As shown in Fig. 6, these two peaks of the oxidized crystalline bovine cytochrome c were much higher than those of reduced form. According to the opinion of

Ungar *et al.* this fact indicates that the ferrocytochrome c is in a more native state (its globular structure is in a more rigid state) than ferricytochrome c. However, it is also possible that crystalline bovine cytochrome c might be more labile at a higher pH 12 in its oxidized form than in its reduced form, because bovine cytochrome c is apt to change its chromatographic species outside the range of pH 5 to 9. Such difference of SGIR between the oxidized and reduced form could not be observed with crystalline yeast cytochrome c. This might be because yeast cytochrome c is more sensitive to a change of pH than bovine cytochrome c and is rapidly and completely modified at pH 12 in either its oxidized or reduced form.

Horse and Pig Cytochrome c—Cytochrome c's from pig and horse gave very similar results to bovine cytochrome c in all of the experiments mentioned above, and were much more like bovine one than to yeast cytochrome c.

DISCUSSION

It was assumed that the milder the purification procedure, the greater is the amount of "native" cytochrome c which can be recovered from a mammalian source compared with "denatured" cytochrome c. Cytochrome c was therefore purified from bovine, horse and pig heart muscle by the various purification procedures involving treatments which were assumed to be mild or drastic to it. After comparisons of the physiological properties of cytochrome c's obtained by these procedures, one fraction was found which was probably the least changed and the cytochrome c was crystallized from it. Crystallization of cytochrome c from the other fractions was very difficult. These experiments lead us to assume that crystalline bovine, horse and pig cytochrome c (fraction 2 in Fig. 1) is the actual form to be found in living cells (native) or at least is the most close to that form.

Though mammalian cytochrome c's were somewhat modified by TCA, the precipitation of cytochrome c by this acid in the presence of the nearly saturated ammonium sulfate as devised by Keilin and Hartree (*2*), if carried out at a neutral pH and a low temperature for only a short time, the procedure is still considered useful for the purifications of bovine, horse and pig cytochrome c but not for that of yeast cytochrome c (*2*). This precipitation is simple and speedy and permits complete recovery of cytochrome ç from a large volume in fairly high state of purity.

During earlier investigations in our laboratory, it was found that bacterial proteinase could not digest some proteins such as bacterial α-amylase (*10*), and triose-phosphate dehydrogenase (*11*, *12*), in their native state (that is when they had enzymic activity). However, after denaturation (that is when they no longer had enzymic activity) they could be digested rapidly by the proteinase. Some enzymes, however, were found to be rapidly digested even in their native state by proteolytic enzyme; Inagaki, Masuda and Okunuki (*13*, *14*) have found that fumarase crystallized from pig liver can be digested in its native state by bacterial proteinase. Moreover, Kunitz and McDonald (*15*) showed that in the absence of its substrate, hexokinase crystallized from baker's yeast can be rapidly digested by trypsin and the proteolytic enzymes present in the yeast cells though there occurred only a little digestion in the presence of substrates. These substrates were found to be protective in the following order; glucose>fructose>mannose. From these facts, it is probable that the susceptibility of a globular protein to digestions by proteolytic enzymes is chiefly influenced by the amino acid sequence of the protein and its globular structure. The amino acid sequence affects the substrate-specificity of the proteolytic enzyme and the globular structure causes steric hinderance to the active area of the enzyme. Though crystalline bovine, horse and pig cytochrome c were much more resistant to digestion by bacterial proteinase and trypsin than crystalline yeast cytochrome c, these mammalian cytochrome c's also were easily digested by the proteinases under the aerobic condition. Like the crystalline yeast cytochrome c, crystalline mammalian cytochrome c's were rapidly digested by bacterial proteinase in their oxidized form, but were hardly digested in their reduced form. This indicates that oxidation and reduction of cytochrome c changes not only the valency of iron in the heme-moiety, but also the inner structure of its protein-moiety. Thus cytochrome c in its reduced form may be protected from the proteinase by an increased steric hinderance, but not by a change of the amino acid sequence.

Bovine, horse and pig as well as yeast cytochrome c (*5*) showed the same absorption spectra in their oxidized and dithionite-reduced forms irrespective of whether they were in the crystalline form or had been modified by treatment such as boiling or with TCA. They could also be oxidized and reduced at almost the same rate by "Green brei" (*16*) and yeast lactic dehydrogenase, respectively. This suggests that it is not possible to decide whether cytochrome c is native or not by study of its oxidation and reduction reactions only, though this might be ascertained using certain physiological reactions involving a change of the inner structure of its protein-moiety resulted from the oxidation and reduction of its heme-moiety.

Cytochrome c is scarcely extractable with water from minced muscles of bovine, horse and pig heart without squeezing with the aid of a compressor unless the extraction is carried out at a higher pH or in the presence of a salt such as ammonium sulfate. The conditions necessary for extraction resemble closely those required for elution of the cytochrome c from a cation exchange resin such as Amberlite XE-64 or Duolite CS-101, though cytochrome c can be more easily eluted in its reduced than in its oxidized form. Since crystalline cytochrome c's are extremely water-soluble and cytochrome c adsorbed on the resins can be oxidized and reduced by additions of a low concentration of chemical oxidant or reductant without being eluted, it seems likely that in the living cells cytochrome c may be fixed on some water-insoluble substance.

SUMMARY

In order to determine "native" cytochrome c, cytochrome c was purified from bovine, horse and pig heart muscle by the various treatment involving both mild and drastic procedures and some physiological properties of the resulting cytochrome c's were compared.

In comparison with the modified cytochrome c's, the most native form of cytochrome c has the lowest ascorbate-oxidizing activity is the least susceptible to digestions by bacterial proteinase and trypsin and has the least affinity for Amberlite XE-64.

Both the native and modified forms are reduced to almost the same extent by yeast lactic dehydrogenase and have the same absorption spectrum in their oxidized and dithionite-reduced forms. Native cytochrome c can be easily crystallized. Crystalline mammalian cytochrome c's are easily digested in their oxidized form by bacterial proteinase, but hardly attacked in their reduced form, indicating that oxidation and reduction of the heme-moiety accompanies a definite change in the protein-moiety of the cytochrome c.

From the properties of the isolated forms of cytochrome c, the state of functional cytochrome c in living cells is discussed.

The authors wish to express their thanks to Messrs. T. Higashi and J. Yamashita for their help and discussion during this investigation.

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STUDIES ON ALKALIGENIC ACID

II. QUANTITATIVE ANALYSIS OF THE COMPONENT AMINO ACIDS AND GLUCOSAMINE BY MEANS OF COLUMN CHROMATOGRAPHY

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In the previous communication (*1*) it has been reported that alkaligenic acid is a special kind of nucleic acid isolated from a certain species of *Alcaligenes faecalis* and contains considerable amounts of sugar-polypeptide complex in a bound form, the latter of which, upon hydrolysis, separates into 9 to 10 amino acids together with glucosamine. The present study deals with the quantitative estimation of its component amino acids and glucosamine according to the method by Moore and Stein (*2*) with slight modification by the writer. At first mention has been made of the modified points, then followed by the description of the analytical data obtained for alkaligenic acid by this technique.

EXPERIMENTS AND RESULTS

A Slightly Modified Moore and Stein Method—The original method by Moore and Stein has been sligtly changed with respect to the reagent solutions for the purpose of simplifying the procedures and minimizing the sample amounts without leading to any appreciable error. Modification of reagents was effected on the three points as in the following.

Modified Reagents—(1) Methyl cellosolve (ethylene glycol monomethyl ether) was replaced by ethylene glycol (b.p. 197°) on account of its higher color yield, its appreciable solvent power and its low cost. Although methyl cellosolve is generally used as a solvent possessing the highest solvent power for hydrindantin, ethylene glycol has also proved to be a solvent satisfactory for this purpose. Moreover, it has been experimentally evidenced that amino acid, *e.g.*, leucine reacts with ninhydrin in ethylene glycol giving an average color yield which is about 11 per cent higher than in methyl cellosolve. The data are shown in Fig. 1.

(2) Water was used simply as a diluent instead of a solvent mixture of equal volumes of *n*-propanol and water. When the quantity of amino acid to be estimated was smaller, water proved to be a satisfactory diluent unlike in the case of larger scale analysis since there occurred neither precipitation of the sodium salt of diketohydrindylidene-diketohydrindamine

nor turbidity formation due to reaction products.

(3) Both stannous chloride and ninhydrin solutions were prepared and kept separately.

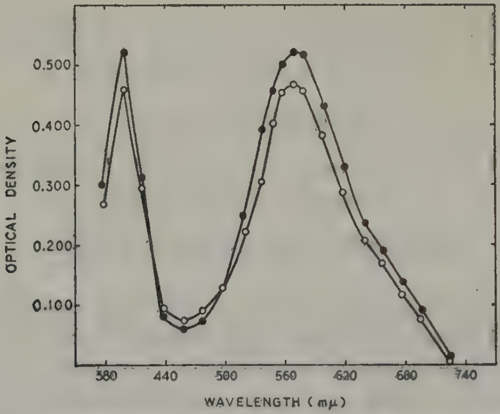

FIG. 1. Comparison of the absorption spectra obtained by the two methods after the reaction of ninhydrin with L-leucine. —○— Moore's method, —●— Modified method.

Stannous Chloride Solution: 2 g. of stannous chloride ($SnCl_2 \cdot 2H_2O$) were dissolved in 124 ml. 0.2 *M* citrate buffer of pH 5.0. This solution could be stored for about 5 days without diminution of the reducing power. Usually, however, a freshly prepared one was used.

Ninhydrin Solution: 1 per cent ninhydrin in a solvent mixture of equal volume of ethylene glycol and 0.2 *M* citrate buffer of pH 5.0. There seemed to be no need of storing these reagents in an atmosphere of nitrogen.

Color Development of Sample by the Modified Method—Due to the minimized sample amount the final volume of the reaction mixture was reduced to 3.5 ml. instead of 6.1 ml., the minimum volume employed by the original method.

1.0 ml. of sample solution adjusted to about pH 5 was placed in a test tube, 0.05 ml. of stannous chloride solution and 1.0 ml. of ninhydrin reagent were added successively and mixed thoroughly by shaking. The tube was heated for 30 minutes in a vigorously boiling water bath. After cooling under running tap water, the colored solution was transferred quantitatively into a graduated test tube with a mark at 3.5 ml., made up with water to the mark and thoroughly mixed by shaking. Readings of the optical density of the colored sample were taken on a Beckman type spectrophotometer at 570 mμ within one hour. The blanks were read against a solution prepared by a similar treatment of the same amount of water instead of a sample solution.

Standard Curve Obtained after the Reaction of Ninhydrin with L-*Leucine by the Modified Method*—To examine the linear relationship between color yield and amino acid content determination was made of the optical densities resulting from the reaction of ninhydrin with L-leucine of varying concentrations. Fig. 2 presents the results, according to which the direct proportionality has been established between color yields and amino acid contents over the wide range of concentration from 0.025 μM (3.27 μg.) to 0.2 μM (26.2 μg.) of L-leucine per 3.5 ml. after final dilution.

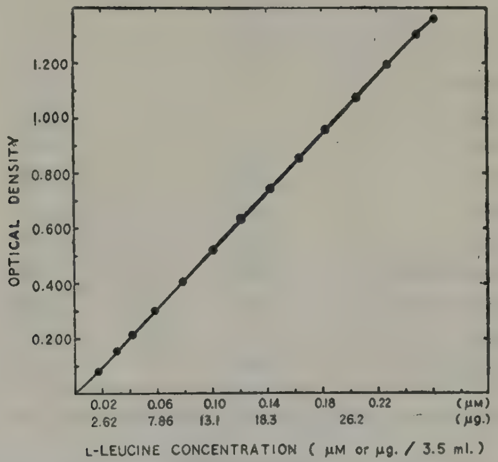

FIG. 2. Standard curve obtained after the reaction of ninhydrin with L-leucine by the modified method.

Color Yields from Amino Acids on Molar Basis Relative to L-*Leucine*—Using 10 amino acids as well as glucosamine found in the hydrolysate of alkaligenic acid quantitative analyses were made to determine the color yields of the individual compounds with ninhydrin. The results obtained are summarized in Table I, in which are presented for comparison those values reported by Moore and Stein. It may be observed that there is a fair agreement between both values for most of the amino acids studied.

Recoveries of Various Amino Acids by Column Chromatography—Quantitative analysis of amino acids by column was conducted following the Moore and Stein method (*3*), namely, the Dowex-50 (250 to 500 mesh) of sodium form was set in a 0.9×100 cm. column and each amino acid was eluted by buffers of progressively increasing pH of citrate buffer (3.42, 4.25, 6.7), $NaHCO_3$-Na_2CO_3 buffer (pH 8.3), $NaHCO_3$-NaOH buffer (pH 9.2) and Na_2CO_3 solution (pH 11.0). The temperature was changed gradually at 37.5°, 50°, 75°, and 25° and the rate of solvent flow through the column was adjusted to about 4 ml. per hour. The effluent was collected in 1 ml. fraction, and the each effluent fraction was brought to about pH 5 with 1 *N* HCl or 1 *N* NaOH and analyzed by the photometric ninhydrin method as mentioned above. By using 1.0 μM of L-leucine as a standard were obtained the recoveries of each 1.0 μM of amino acids by column chromatography. The average results from 10 chromatograms obtained by the integration of the curve in chromatograms

TABLE I

Color Yields from Amino Acids on Molar Basis Relative to L-Leucine

Determined on 0.1 μM of each amino acids; heating time, 30 minutes; read at 570 mμ.

Amino acids	Color yield	
	Results obtained	Moore and Stein*
Leucine	1.00	1.00
Isoleucine	1.00	1.00
Phenylalanine	0.96	0.91
Valine	1.01	1.00
Alanine	0.99	0.99
Glycine	1.04	1.01
Serine	0.97	0.97
Glutamic acid	0.99	1.05
Aspartic acid	0.99	0.93
Lysine	1.13	1.12
Glucosamine	0.95	1.00

* According to the works by Moore and Stein (*2*, *3*).

TABLE II

Recoveries of Various Amino Acids by Column Chromatography

The amino acids used for study as an equimolar mixture are those to be found in alkaligenic acid.

Amino acids	Average recovery and deviation from mean	
	Results obtained (10 chromatograms)	Moore and Stein (12 chromatograms)
Leucine	100.3±1.0	100.6±2.1
Isoleucine	101.3±1.8	101.0±1.4
Phenylalanine	96.5±1.3	98.2±1.4
Valine	98.8±3.0	100.8±2.9
Alanine	100.1±2.8	100.7±2.2
Glycine	99.8±3.0	99.2±2.8
Serine	99.0±2.5	100.4±2.6
Glutamic acid	97.5±2.5	96.7±2.1
Aspartic acid	99.4±2.0	99.6±2.7
Lysine	82.0±6.0	82.0±5.0
Glucosamine	103.5±2.8	

of the various amino acids are summarized in Table II.

As can be seen in the above table recoveries are as high as over 98 per cent and deviations from mean values are less than ±3 per cent for the majority of the amino acids being studied. These findings are quite in accord with those by Moore and Stein cited in the same table.

Quantitative Component Analysis for Sugar-Polypeptide Complex of Alkaligenic Acid- -Since the sugar-polypeptide fraction was unable to be isolated from alkaligenic acid, the analytical work was performed using alkaligenic acid itself

19.34 mg. of alkaligenic acid together with 1.9 ml. of redistilled 6 *N* HCl were sealed in a glass tube and heated for 24 hours in a boiling water bath.

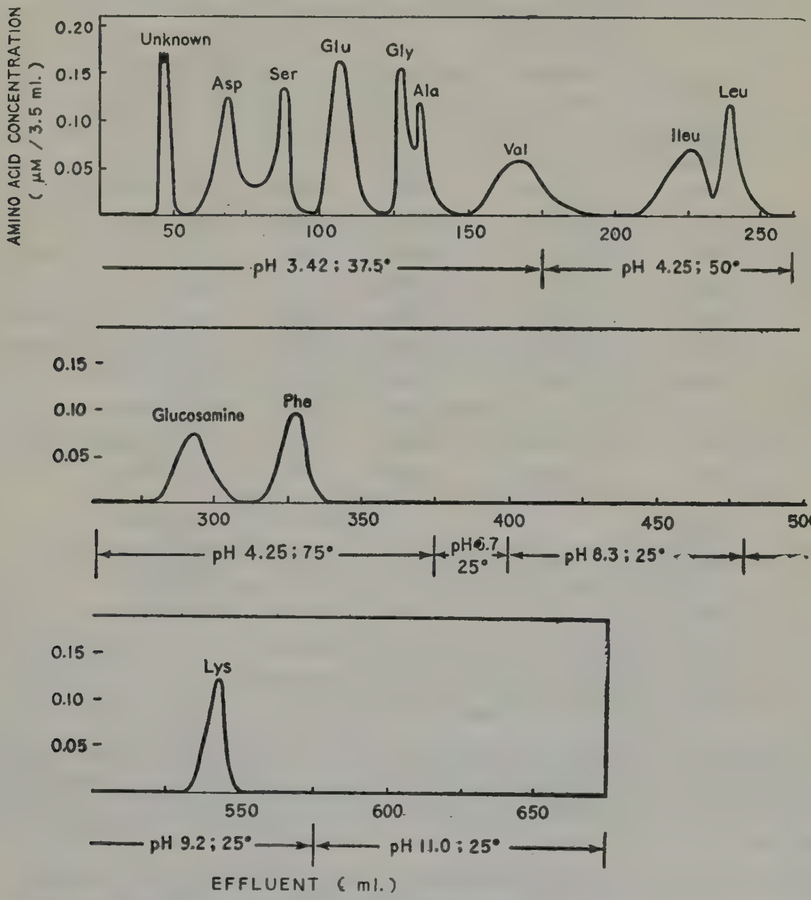

FIG. 3. Chromatographic analysis by column of amino acids and glucosamine contained as sugar-polypeptide in alkaligenic acid.

The hydrolysate was centrifuged to remove the resulting precipitate and the latter washed 3 times with 0.5 ml. portions of distilled water. The supernatant fluid combined with washings was vacuum evaporated to dryness several times with water over P_2O_5 and NaOH to remove excess HCl. The final dried residue was dissolved in 0.5 ml. of citrate buffer of pH 3.0 and placed on the chromatographic column. Fractionation of each 1 ml. effluent was carried out by utilizing an automatic siphon-balance fraction collector manufactured by Toyo Filter Paper Co., Ltd. Fig. 3 illustrates the chromatographic pattern obtained according to the procedure mentioned above. In Table III the whole data are expressed in terms of figures involved in the evaluation of the chromatogram.

TABLE III

Quantitative Analysis of Individual Amino Acids and Glucosamine Contained as Sugar-Polypeptide Complex in Alkaligenic Acid

Amino acid	Ratio of peak area*	Conversion factor**	Amino acid content		
			μM***	μg.	*per cent*
Unknown	1.03	—	—	—	—
Asp	1.03	0.984	1.05	140	0.72
Ser	0.94	0.960	0.98	103	0.53
Glu	1.41	0.965	1.46	215	1.11
Gly	1.12	1.030	1.09	82	0.42
Ala	0.65	0.990	0.66	59	0.31
Val	1.00	0.998	1.00	117	0.61
Ileu	1.03	1.010	1.02	134	0.69
Leu	1.03	1.000	1.03	135	0.70
Phe	0.94	0.927	1.01	167	0.86
Lys	1.03	0.927	1.11	162	0.84
Sum			10.41	1314	6.79
Glucosamine	1.00	0.984	1.02	183	0.95
Total			11.43	1497	7.74

* 1.0 μM of L-leucine area = 1.00.
** Conversion factor = Color yield × per cent recovery.
*** Ratio of peak area/conversion factor.

The above table indicates that each amino acid and glucosamine exist in alkaligenic acid in quantities of almost equal molecule except for glutamic acid (1.46 μM) and alanine (0.66 μM). It is interesting to note that an unidentified sharp peak has appeared in the 46th effluent fraction as a first runner just before aspartic acid.

DISCUSSION

It has been experimentally demonstrated that the present amino acid assay method achieved by slight modification of the Moore and Stein method is able to give satisfactory results. The qualitative study made in the previous work pointed out that 9 to 10 amino acids were detected in the hydrolysate of alkaligenic acid by two-dimentional paper chromatography. According to the quantitative analysis carried out by the method stated above, it has been revealed that each of the 10 amino acids as well as glucosamine ezist in an approximately equimolar proportion with the exception that glutamic acid was found in a higher and alanine in a lower quantity.

Alkaligenic acid has been previously reported to contain as much as 8.5 per cent sugar-polypeptide complex, the value of which was estimated on the hydrolysates by the photometric ninhydrin method employing leucine as a standard. Accordingly, this value includes, in addition to amino acids, glucosamine and an unknown ninhydrin positive substance referred to above. As may be seen in Table III, the amino acid content obtained by summing up the contents of individual amino acids amounts to 6.79 per cent, and if glucosamine is taken into calculation, this value increases to 7.74. The remaining number left after subtraction of 7.74 from 8.5, namely, 0.8 may be accounted for the unidentified ninhydrin positive substance.

Concerning the chemical nature of this ninhydrin positive substance of which peak emerged at the 46th fraction tube, no information is available at the present stage. The literatures indicate that urea or taurine is likely to appear in that area of the chromatogram given above. Both of them, however, were rejected by paper chromatographic analyses which were conducted with sample solutions from the corresponding effluent fraction. Due to the obscure nature of this substance, therefore, uncertainty of the ninhydrin color yield, its recovery estimated at 0.8 would not be an exact one.

SUMMARY

Quantitative analysis for the amino acids and glucosamine assumed to occur as sugar-polypeptide complex in alkaligenic acid was conducted following the method by Moore and Stein with slight modification. It has been evidenced that glucosamine as well as ten amino acids including aspartic acid, serine, glutamic acid, glycine, alanine, valine, isoleucine, leucine, phenylalanine and lysine are present in an approximately equimolar proportion with the exception that glutamic acid exists to a higher and alanine to a lower extent. In addition, an unidentified ninhydrin positive substance was found as a sharp peak appearing ahead of the aspartic acid peak in the chromatographic pattern.

The auther wishes to express her gratefulness to Prof. Akashi for his kind encouragement and advice throughout this investigation.

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THE OXIDATION SYSTEMS OF MITOCHONDRIAL PARTICLES FROM ASPERGILLUS ORYZAE*

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Recently, many papers have been published on the oxidation systems of animal mitochondria (*1*,*2*). In fungi, it has also been reported that *Aspergillus niger* (*3*, *4*) has particles which are stained with Janus green B and coloured by Nadi reagent, and yeast (*5*) has particles possessing the activity of electron transport and oxidation of TCA**-cycle intermediates. In a previous report (*6*), it was shown that the mitochondrial particles from *Aspergillus oryzae* stain aerobically with Janus green B and possess a type of ATP-ase which increases in activity as the particles age.

In the present paper, experiments will be described showing that fungal mitochondria also oxidized components of TCA-cycle and DPNH. It is also shown that the stimulating effect on the oxidation of DPNH of cyanide in extremely low concentration may be attributed to chelation of some cation(s) which is bound to the particles and normally maintains a low rate of oxidation.

EXPERIMENTAL

The mycelial mats of *Aspergillus oryzae* after culture on Pfeffer's medium were homogenized with 1 M of sucrose containing 0.01 M $MgCl_2$ and 0.1 M Tris-buffer at pH 7.5, and were fractionated as described previously (*6*). A slight modification of the differential centrifugation was made as follows; debris was precipitated from mitochondrial suspensions by centrifugation for 5 minutes at $500 \times g$, from microparticles by centrifugation for 20 minutes at $10,000 \times g$ and from the soluble fraction by centrifugation for 30 minutes at $20,000 \times g$.

Mitochondria and microparticles were suspended in 0.5 M of sucrose containing 0.005 M $MgCl_2$ and 0.05 M Tris-buffer at pH 7.5. The protein nitrogen content per ml. was approximately 15 μg. in the mitochondrial suspension, 30 μg. in the suspension of microparticles, and 40 μg. in the supernatant. When the mitochondria were washed with 0.25 M

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* A preliminary report of this work was presented at the Annual Meeting of the Botanical Society at Sapporo, in July, 1956.

** The following abbreviations were used: DPN, diphosphopyridine nucleotide: DPNH, reduced diphosphopyridine nucleotide: Tris, trishydroxymethyl aminomethane: ATP, adenosine triphosphate: EDTA, ethylenediamine tetraacetate: TCA, tricarboxylic acid: DCA, dicarboxylic acid.

sucrose, their oxidative activity decreased.

The oxidation of DPNH was followed in a Simadzu photoelectric spectrophotometer, type QB-50, at room temperature (approximately 25°). The reaction was started by addition of enzyme, and after one minute the optical density was measured at 340 mμ. The decrease of optical density was measured after one minute.

Oxidation of components of TCA-cycle, were studied spectrophotometrically by measuring the rate of reduction of added cytochrome c or 2,6-dichlorophenol indophenol. Initiation and measurement of the reaction, were performed as described above. Since the rate of decrease in optical density of DPNH at 340 mμ and of 2,6-dichlorophenol indophenol at 600 mμ, and the increase in optical density of reduced cytochrome c at 550 mμ were linear for several minutes, the rate of the reaction was calculated from the initial slope.

DPNH was prepared according to the method of Le Page (*7*). DPN obtained from baker's yeast was purified approximately 90 per cent (*8*) and reduced in a buffered solution by addition of a minimal amount of hydrosulfite. After aeration, the DPNH solution was adjusted to pH 7.5 and used for the present experiments. Crystalline cytochrome c obtained from bovine heart muscle (*9*) was reduced by addition of a minimal quantity of hydrosulfite as described above and used at pH 7.5. Co-carboxylase was kindly supplied by Mr. Wakisaka (Sionogi Medical Drug Co. Ltd.).

RESULTS

(*A*) *Oxidation of TCA-Cycle Intermediates*—It is well known that energy supplying reactions, such as TCA-cycle, occur in mitochondrial particles in animal cells. It is, therefore, natural to assume that the oxidation of members of TCA-cycle will also occur in mitochondrial particles from the mycelium of *Asp. oryzae.* We have studied this by measuring the decrease of optical density at 600 mμ during reduction of 2,6-dichlorophenol indophenol, and the increase in optical density at 550 mμ during reduction of cytochrome c in the presence of cyanide, under suitable conditions. Table I shows that succinate, α-ketoglutarate, *cis*-aconitate, malate, citrate, fumarate, and pyruvate were oxidized by the suspension of mitochondria. Since succinate was always oxidized rapidly without DPN added and its oxidation was completely inhibited by an equimolar concentration of malonate, it is possible that the enzymes known to participate in TCA-cycle function in the fungal mitochondrial particles.

Pyruvate however did not reduce externally added cytochrome c even in the presence of several co-factors (co-carboxylase, ATP, DPN) and various substances added as sparker (α-ketoglutarate, citrate, malate, acetate, glutamate, fumarate and *cis*-aconitate), although in the course of its oxidation it reduced 2,6-dichlorophenol indophenol rapidly and steadily. The oxidation of pyruvate did not require DPN, and its oxidation was not inhibited, but instead strongly stimulated by 10^{-3} *M* cyanide. Cyanide also stimulated DPNH-oxidation as described below. From the result, it is reasonable to assume that pyruvate is oxidized by a pathway other than the cyanide-sensitive cytochrome system.

With the exception of succinate and pyruvate, the oxidation of the other substrates tested required DPN. Oxidation of α-ketoglutarate was stimulated by addition of co-factors such as co-carboxylase and ATP. Acetate was not however oxidized under any conditions tested, even in the presence of co-factors and sparkers. Succinate, α-ketogluterate, *cis*-aconitate and pyruvate were shown to be oxidized by microparticles, although at a lower rate than by mitochondrial particles.

TABLE I

Oxidation of Components of TCA-Cycle by Mitochondria and Microparticles

		Mitochondria		Microparticles
Substrate added	Addition (+) or omittion (−)	ΔE_{550}/*min./mg. of nitrogen*	ΔE_{600}/*min./mg. of nitrogen*	ΔE_{600}/*min./mg. of nitrogen*
Succinate		3.07	2.20	1.20
„	+Malonate	0.00	—	—
α-Ketoglutarate		0.82	0.73	—
„	+Co-factors*	1.43	—	0.34
„	−DPN	0.00	—	—
cis-Aconitate		1.10	1.20	0.20
„	−DPN	0.00	—	—
Malate		1.12	1.10	—
„	−DPN	0.00	—	—
Citrate		0.66	—	—
Fumarate		0.36	—	—
Glutamate		0.12	—	—
Acetate		0.00	0.00	—
Pyruvate		0.00	2.67	1.10
„	−DPN	—	2.70	—
„	−DPN and −KCN	—	1.47	—

Reaction mixture: 0.5 ml. of Tris-buffer (0.2 M, pH 7.5), 0.3 ml. of DPN (0.2 mg. per ml.,), 0.3 ml. of $10^{-2}\,M$ KCN, 0.1 ml. of 0.1 M $MgCl_2$, 0.1 ml. of 0.1 M $MnCl_2$, 0.3 ml. of cytochrome c (15×10^{-4} μ moles), or 0.2 ml. of 2,6-dichlorophenol indophenol (0.2 μ moles), 0.3 ml. of substrate (30 μ moles), 0.3 ml. of mitochondrial suspension (4.5 μg. of nitrogen), or 0.3 ml. of microparticles (9.0 μg. of nitrogen). The total volume 30 ml.

* 150 μg. of co-carboxylase and 3 μ moles of ATP in 0.3 ml.

(*B*) *Electron Transport System*—When DPNH was added as a substrate to a suspension of mitochondria, a distinct decrease in optical density at 340 mμ was observed (Fig. 1). The addition of cytochrome c did not increase the rate of the oxidation, suggesting that cytochrome c did not participate in the oxidation of DPNH in the mitochondria. Fig. 1 shows however that oxidation of DPNH was strongly inhibited by $10^{-3}\,M$ KCN and partially inhibited by concentrations of $10^{-4}\,M$ and $10^{-5}\,M$. But it was found that not only the oxidation of reduced cytochrome c, but also the reduction of added cytochrome c were catalyzed by a suspension of mitochondria. The oxidation reaction was completely inhibited by $10^{-3}\,M$ KCN. From these results, it appeared to us that the suspension of mitochondria contained cytochrome c oxidase and cytochrome c reductase.

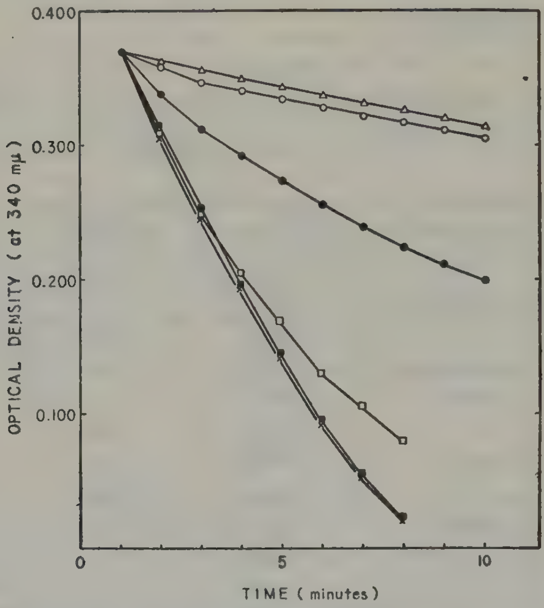

FIG. 1. The effect of cyanide and cytochrome c on the oxidation of DPNH by suspension of mitochondria.

Complete system: 0.5 ml. of Tris-buffer (0.2 M, pH 7.5), 0.3 ml. of DPNH (approximately $130\,\mu$g.), 0.3 ml. of mitochondrial suspension ($4.5\,\mu$g. of protein nitrogen), water to 3.0 ml. The reaction was carried out at room temperature. The control cuvette contained no DPNH. —×—, Complete system. Additions to complete system: —○—, $10^{-3}\,M$ KCN; —●—, $10^{-4}\,M$ KCN; —□—, $10^{-5}\,M$ KCN; —■—, Cytochrome c ($15\times10^{-4}\,\mu$moles). —△—, Mitochondrial suspension boiled for 30 minutes, other conditions being as in complete system.

Since it can be seen with a microspectroscope that the absorption bands of cytochromes b, c and a disappear reversibly in a heavy suspension of mitochondria of *Asp. oryzae*, it is reasonable to conclude that these mitochondria can catalyze the oxidation of DPNH by O_2 through cytochrome c. In this case, the cytochrome b band is the strongest and that of cytochrome a is weak.

Not only in suspensions of microparticles but also in the soluble fraction DPNH is oxidized by O_2, although at slower rates than by suspensions of mitochondria. It was shown that the oxidation of DPNH by microparticles was stimulated significantly by a low concentration of cyanide (Fig. 2), whereas, as in the case of mitochondrial suspensions, the oxidation was inhibited by 10^{-3} *M* cyanide. DPNH oxidation by the soluble fraction, on the contrary, was scarcely inhibited by addition of KCN (Table II). It is

TABLE II

Comparison of Activities on Fractions

Activities	KCN	Mitochondria ΔE	Mitochondria %	Microparticles ΔE	Microparticles %	Soluble fraction ΔE	Soluble fraction %
(A) Oxidation of DPNH	−	14.22	100	3.56	25	1.61	11
	+	0.43	3	0.14	1	1.13	8
(B) Oxidation of cytochrome c	−	4.20	100	0.93	22	—	
	+	0.04	1	0.01	0	—	
(C) Reduction of cytochrome c	+	4.00	100	0.72	18	—	

ΔE decreased at 340 mμ (A), decreased at 550 mμ (B), and increased at 550 mμ (C) are expressed per minute per mg. of protein nitrogen.

(A): 0.5 ml. of Tris-buffer (0.2 *M*, pH 7.5), 0.3 ml. of DPNH (130 μg.), 0.3 ml. of mitochondrial suspension (4.5 μg. of protein nitrogen) or microparticles (9.0 μg. of protein nitrogen), or 0.9 ml. of soluble fraction (36.0 μg. of protein nitrogen), water to 3.0 ml. KCN was added at 10^{-3} *M*.

(B): As in (A) except that 0.3 ml. of reduced cytochrome c (15×10^{-4} μ moles) was added instead of DPNH.

(C): As (A) except that 0.3 ml. of cytochrome c (15×10^{-4} μ moles) and 0.3 ml. of 10^{-2} *M* KCN were added.

TABLE III

Increase in Rate of DPNH Oxidation by KCN and EDTA

Addition	Mitochondria			Microparticles
	$-\Delta E_{340}$/min./mg. of protein nitrogen			
	Concentration of protein nitrogen in the reaction mixture			
	4.5 μg.	2.3 μg.	1.5 μg.	9.0 μg.
None	14.41	14.22	17.31	2.78
10^{-4} *M* KCN	5.10	—	13.35	1.22
10^{-5} *M* KCN	11.35	15.14	28.68	3.70
10^{-6} *M* KCN	13.40	16.00	36.87	4.78
10^{-2} *M* EDTA	—	—	—	4.45
10^{-3} *M* EDTA	—	—	37.89	3.89
10^{-4} *M* EDTA	—	—	20.04	3.23

Conditions as in Table II (A) except that 0.3 ml. of KCN or EDTA was added to the reaction mixture at the various concentrations indicated.

assumed that in the soluble fraction the oxidation is catalyzed by a cyanide-insensitive DPNH oxidase.

(*C*) *Stimulation of DPNH Oxidation by Cyanide*—As described above, DPNH oxidation by a suspension of microparticles was inhibited by cyanide at a high concentration (10^{-3} *M*), but was strongly stimulated at a low concentration (Fig. 2 and Table III). The stimulatory effect of KCN on the oxidation of DPNH was also observed in a diluted suspension of mitochondria. When the amount of mitochondria in the reaction mixture was equivalent to 4.5 μg. of protein nitrogen, the rate of oxidation was not increased by cyanide. When the mitochondria were at an amount of 2.3 μg. of the nitrogen, the increase of oxidation was just detectable, and at an amount of 1.5 μg. of the nitrogen, the rate of oxidation was increased about two-fold by addition of 10^{-6} *M* KCN.

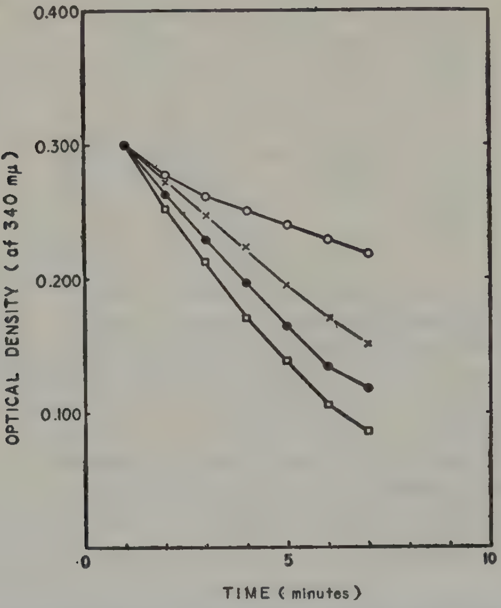

FIG. 2. Stimulation by cyanide of DPNH oxidation in a suspension of microparticles.

Condition as in Fig. 1 except that a suspension of microparticles (9 μg. of protein nitrogen) added. —×—, Complete system. Additions to complete system: —○—, 10^{-4} *M* KCN; —●—, 10^{-5} *M* KCN; —□—, 10^{-6} *M* KCN.

Since cyanide is known to be not only an inhibitor of the oxidase, but also a chelating reagent for heavy metals such as Fe or Cu, the effect of EDTA, a typical chelating reagent, on the oxidation of DPNH by microparticles was examined. As shown in Table III, the rate of oxidation was stimulated by addition of EDTA to suspension of microparticles and diluted suspension of mitochondria. The concentration of EDTA necessary to double the rate of oxidation was $10^{-3}\,M$ in a suspension of mitochondria containing 1.5 μg. of protein nitrogen in the reaction mixture and $10^{-2}\,M$ in the case of microparticles. Since the effect of cyanide or EDTA on the oxidation of DPNH was not observed in the soluble fraction, it seems likely that these effects must be related to particulate structures.

It may therefore be assumed that some cations bound to the mitochondrial particles maintain a low level of DPNH oxidation, and stimulation of oxidation is brought about by chelation of these cations. A study was made of various cations which inhibit oxidation of DPNH. These were added to a reaction mixture at pH 7.5 and 6.0. The oxidation of DPNH by mitochondrial suspensions and by suspensions of microparticles was strongly inhibited by the presence of $2\times10^{-4}\,M$ and $10^{-4}\,M$ $FeSO_4$, as shown in Table IV.

TABLE IV

Inhibition of DPNH Oxidation by Iron

Fraction	pH	Addition	$-\Delta E_{340}$/*min./mg. of protein nitrogen*	Percentage of inhibition
(A) Mitochondria	7.5	None	12.42	
	„	$10^{-4}\,M$ $FeSO_4$	3.78	70
	„	$2\times10^{-4}\,M$ „	2.00	84
(B) Mitochondria	6.0	None	4.37	
	„	$2\times10^{-4}\,M$ $Fe_2(SO_4)_3$	2.22	49
(C) Microparticles	7.5	None	4.88	
	„	$2\times10^{-4}\,M$ $FeSO_4$	1.11	77
(D) Microparticles	„	None	3.38	
	„	$10^{-5}\,M$ $FeSO_4$	3.11	6
	„	$10^{-4}\,M$ „	0.72	78
(E) Microparticles	6.0	None	3.56	
	„	$4\times10^{-5}\,M$ $FeSO_4$	2.56	28
	„	$10^{-4}\,M$ „	2.22	39
	„	$2\times10^{-4}\,M$ „	2.12	41
	„	$4\times10^{-4}\,M$ „	1.55	57
	„	$2\times10^{-4}\,M$ $Fe_2(SO_4)_3$	2.10	41

Conditions as in Table II (A) except that 0.3 ml. of iron compounds at various concentrations were added and the pH of some reaction mixtures (B and E) was 6.0 (0.2 *M* phthalate buffer).

A lactic dehydrogenase system from yeast was used to determine the residual amount of DPNH in a reaction mixture inhibited by Fe ions. In one such experiment when the amount of DPNH oxidized by a suspension of mitochondria ($-\Delta E_{340}$/mg. of protein nitrogen, at 15°) was 38.2 and the amount oxidized in the presence of $5\times10^{-4}\,M$ $Fe_2(SO_4)_3$ was 4.4. The residual amount of DPNH was adout 80.0 (ΔE_{340} decreased by lactic dehydrogenase system) in the control reaction and about 128.0 in reaction mixture contained $Fe_2(SO_4)_3$. This showed therefore that a larger amount of added DPNH remained in the reaction mixture containing $Fe_2(SO_4)_3$. The inhibition of DPNH oxidation by 10^{-4} *M* $FeSO_4$ was reversed by addition of KCN or EDTA (Table V). For example, 78 per cent of the activity was recovered by addition of 10^{-4} *M* KCN and 58 per cent by 10^{-5} EDTA. The rate of oxidation after addition of a high concentration of EDTA was greater than in the absence of added Fe ions.

TABLE V

Recovery of DPNH Oxidation in the Suspension of Microparticles Inhibited by Iron

Addition	$-\Delta E_{340}$/ *min./mg. of protein nitrogen*	Percentage of original activity
None	3.34	100
10^{-4} *M* $FeSO_4$	0.72	22
10^{-4} *M* $FeSO^4$ + 10^{-5} *M* KCN	0.98	29
„ + 10^{-4} *M* KCN	2.79	83
„ + 10^{-3} *M* KCN	0.45	14
„ + 10^{-6} *M* EDTA	1.78	53
„ + 10^{-5} *M* EDTA	2.23	67
„ + 10^{-4} *M* EDTA	4.45	133
„ + 10^{-3} *M* EDTA	6.65	200

Conditions as in the Table II (A) except that 0.3 ml. of 10^{-3} *M* $FeSO_4$ and 0.3 ml. of KCN or EDTA in various concentrations were added.

DISCUSSION

Mitochondrial particles isolated from *Aspergillus oryzae* stain with Janus green B and possess adenosine triphosphatase activity which increases with ageing. They can oxidize components of TCA-cycle through the cytochrome system. The microparticles are reddish brown and in suspension are opalescent. The particles may represent a fraction containing microsomes, although there are also some small mitochondria which have the ability to oxidize components of TCA-cycle.

Ungerminated conidio-spores of *Asp. oryzae* oxidized monosaccharides and fatty acids, but not components of TCA-cycle, and these oxidations are not inhibited by cyanide, monoiodoacetate or carbon-monooxide*. From the results, it is reasonable to conclude that the oxidative pathway of the conidio-spores does not involve the TCA-, or DCA-cycle, and that during germination the conidio-spores gain the ability to oxidize components of the TCA- (and DCA-) cycle, and that the cytochrome system becomes apparent. We have been interested in the results obtained in the present studies with *Asp. oryzae* from the point of view of the comparative physiological properties of mitochondria.

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* Unpublished data of Okunuki, and Iwasa.

According to the report of Humphreys and Conn (*10*), the oxidation of DPNH by mitochondria isolated from white lupine requires the addition of cytochrome c, but that of our preparation did not, since cytochrome c is present in our preparation at a level which is distinctly visible with a hand-spectroscope.

Addition of cyanide to the reaction mixture caused marked stimulation of DPNH oxidation. Since EDTA produced effects similar to those of cyanide, it is assumed that some cations bound to the particles are capable of suppressing that of oxidation and the suppression is reversed when they are removed from the particles by chelation with cyanide. The cations suppressing the rate of oxidation may be Fe^{++} or Fe^{+++} in the culture medium, because oxidation of DPNH was inhibited by addition of Fe^{++} ($FeSO_4$) or Fe^{+++} ($Fe_2(SO_4)_3$). Lehman and Nason (*11*) reported that a preparation of DPNH-cytochrome c reductase from rat skeletal muscle was completely inhibited by 5×10^{-4} *M* Fe^{++} but not by the same concentration of Fe^{+++}. On the other hand, it was found that oxidation by mitochondria of external DPNH (added to a reaction mixture) was not accompanied by phosphorylation (*12*, *13*). Moreover the oxidation was not inhibited by amytal and antimycin A. From these results it was suggested that external DPNH, even in intact mitochondria, was oxidized by transfer of electron to oxygen, probably through a flavoprotein (*e.g.* DPNH-cytochrome c reductase). It is reasonable to conclude that some properties of the oxidation of external DPNH by mitochondria of *Asp. oryzae* found in these experiments may differ from those of internal DPNH produced by dehydrogenase.

SUMMARY

1. Mitochondria isolated from mycelia of *Aspergillus oryzae* catalyzed the oxidation of exogenous DPNH and components of TCA-cycle. These oxidations were carried out through cytochrome c, which was observed in mitochondria with a hand-spectroscope.

2. Contrary to the current conception, it was not demonstrated that external cytochrome c and DPN are essential for the oxidation of pyruvate in the case of mitochondria of *Asp. oryzae*. However, 2,6-dichlorophenol indophenol was reduced rapidly in the presence of pyruvate. The oxidation of pyruvate by mitochondria was strongly stimulated rather than being inhibited by 10^{-3} *M* cyanide.

3. DPNH oxidation was stimulated by EDTA and by a low concentration of cyanide. It is concluded from these results that the stimulation is accompanied by chelation of some cations which may be bound to the particles and normally maintain a low rate of oxidation.

4. Fe^{++} and Fe^{+++} ions inhibited the oxidation of DPNH strongly and their inhibition was reversed by addition of either cyanide at the low concentration or of EDTA.

5. The properties of the mitochondria were compared with those of particles and the soluble fraction.

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THE STRUCTURE OF CYTOCHROME C

IV. TREATMENT WITH CARBOXYPEPTIDASE AND AMINOPEPTIDASE*

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In the classical investigation of cytochrome c, Tsou (*1*) treated the protein with proteases to investigate to what extent a modification in the protein of the enzyme would be reflected in the catalytic and other properties. He observed the disappearance of activity as well as the appearance of autoxidizability and affinity towards carbon monoxide as the digestion proceeded. He could not obtain any fragment of smaller molecular weight which was catalytically active in biological systems.

Recently several investigators examined the biological activities of some enzymes and hormones after the release of their carboxyl or amino terminal amino acids (*2*). Crystalline carboxypeptidase of pancrease extract and leucine aminopeptidase of swine kidney were used for the purpose. The retention of biological activity after the removal of several amino acids residues was reported.

In the previous investigations (*3*, *4*), authors treated cytochrome c with acetyl anhydride or *O*-methyl-isourea, to find out the effects of chemical modification on its biochemical properties. In this paper are presented data on the digestion of cytochrome c with carboxypeptidase and aminopeptidase. The catalytic activity in a succinic oxidase remained unchanged until more than seven molecules of carboxyl terminal amino acids were splitted.

MATERIALS AND METHODS

Cytochrome c—Cytochrome c was prepared from horse heart and purified chromatographically as reported in the previous paper (*3*).

Carboxypeptidase—Crystalline carboxypeptidase was prepared from the press juice of beef pancreas by the modified method of Neurath (*5*). It was recrystallized more than five times with addition of diisopropylfluorophosphate (DFP).

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* A part of this study has been reported at the International Symposium on Enzyme Chemistry, October 1957, Tokyo

Leucine Aminopeptidase—Leucine aminopeptidase was prepared from swine kidney according to the method of Smith (*6*) and purified further using zone-electrophoresis on starch column.

Digestion with Carboxypeptidase—Cytochrome c (100 mg. in 5 ml. of distilled water) was added to a solution of carboxypeptidase (10 mg. in 20 ml.). The solution of carboxypeptidase was prepared as follows; the enzyme (10 mg.) was dissolved in 2 ml. of 10 per cent lithium chloride and diluted to 20 ml. after adjusting to pH 8.0 by lithium hydroxide. Diisopropylfluorophosphate solution (0.05 ml. of 0.5 M DFP in isopropanol) was added to inhibit the contaminating endopeptidases. The mixture was incubated at 37° with occasional adjustment of pH with lithium hydroxide.

The aliquots were pipetted out at intervals. Each aliquot was divided into two portions. The first portion was stocked in frozen state for the estimation of catalytic activity in a succinic oxidase system and the affinity towards carbon monoxide. The second portion (2 ml.) was used for the determination of free amino acids.

In some experiments the control experiment was carried out to estimate the release of free amino acids from carboxypeptidse (autodigestion). The carboxypeptidase solution was incubated at 37° without addition of cytochrome c and the aliquots were removed for the determination of free amino acids.

Digestion with Leucine Aminopeptidase—Cytochrome c was added to the solution of leucine aminopeptidase in 0.005 M veronal buffer of pH 8.5, containing 0.005 M $MgCl_2$ (cytochrome c : leucine aminopeptidase = 100 : 1 mole/mole) and incubated at 37°. Aliquots were pipetted out at intervals for the estimation of amino acids liberated.

Analysis of Amino Acids Liberated—The mixtures of cytochrome c and carboxypeptidase were allowed to react with 2,4-dinitrofluorobenzene. The dinitrophenylated amino acids were separated from the precipitated proteins and estimated spectrophotometrically at 420 mμ in acidic medium. A standard alanine solution was similarly treated and used for the calculation of moles of amino acids liberated. In some experiments, dinitrophenyl amino acids were separated using paper chromatography (*7*) and the eluates were analysed spectrophotometrically for individual amino acids.

In the experiments using leucine aminopeptidase, paper chromatography (butanol : acetic acid : water = 4 : 1 : 2) and paper electrophoresis (pH 6) (*8*) were used for the separation of histidine which was assumed to be situated at N-terminal. The papers were sprayed with diazo reagent of Pauly for the detection of histidine.

Properties of Carboxypeptidase-Digested Cytochrome c—Estimation of catalytic activity of digested cytochrome c in a succinic oxidase system and the affinity of the treated pigment towards carbon monoxide were carried out as reported in the previous paper (*3*).

RESULTS AND DISCUSSION

Digestion with Carboxypeptidase—The appearance of free amino acids was observed during the course of the digestion of native cytochrome c with carboxypeptidase. Fig. 1 shows an example of the release of amino acids from cytochrome c as well as the release of amino acids from carboxypeptidase itself without the addition of the substrate. Although several amino acids noted below appeared simultaneously at the initial short term of incubation, the appearance of glutamic acid, which was indicated to be a C-terminal amino acid by hydrazinolysis (*8*), preceded that of other amino acids in earlier period. An appreciable increase of valine and leucine, estimated as a mixture of the two, was observed. Serine and lysine followed them. Amino acids liberated in the first 24 hours were as follows: valine and leucine, serine (including asparagine), lysine, glutamic acid, phenylalanine, threonine, alanine and glycine.

Properties of Digested Cytochrome c—Optical density at 550 mμ in the reduced state, affinity towards carbon monoxide and the catalytic activity as an electron carrier of the digested cytochrome c are given in Table I. Affinity towords carbon monoxide as estimated by the method of Tsou did not increase after the digestion. The catalytic activity of the protein in a succinic oxidase system was unaltered by carboxypeptidase digestion. The only change observed was the acquisition of a relative instability against heat. The modified cytochrome c coagulated at the temperature in a boiling water bath, while the native one did not show any change under the same condition.

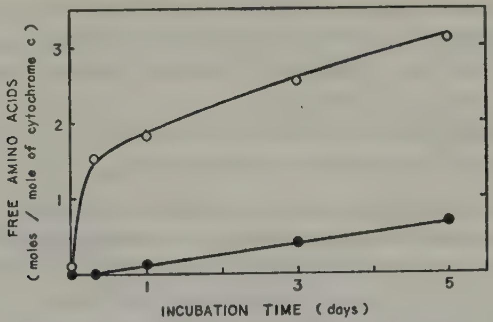

FIG. 1. Release of amino acids from cytochrome c by carboxypeptidase digestion. —○—, From cytochrome c; —●—, Autodigestion.

TABLE I

Digestion of Cytochrome c with Carboxypeptidase

Days of incubation	Moles of amino acids released	Optical density at 550 mμ in the reduced state	Affinity towards carbon monoxide (%)	Catalytic activity in a succinic oxidase system (%)
0	0.4	0.272	11.1	100
1	3.5	0.270	8.9	95
3	4.8	0.259	8.8	108
5	6.3	0.260	9.4	103
7	6.15	0.273	10.1	102
9	7.7	0.281	17.0	110

Digestion with Leucine Aminopeptidase—An appearance of free histidine was pursued in the digest of cytochrome c by leucine aminopeptidase, because histidine has been known to be the N-terminal amino acid of equine cytochrome c (*9*), but neither paperelectrophoresis nor paper chromatography revealed the splitting of histidine or other amino acids. This finding supports the hypothesis of Margoliash, that N-terminal histidine is coordinated to heme and concealed in a crevice of protein.

SUMMARY

More than seven moles of amino acids were liberated from the C-terminal of cytochrome c by the treatment with carboxypeptidase. The catalytic activity of the protein in a succinic oxidase system was retained through out the course of digestion.

Leucine aminopeptidase prepared from swine kidney did not split N-terminal amino acid from native cytochrome c.

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SPLITTING OF CATALASE MOLECULE BY ALKALI TREATMENT

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In 1938, Sumner and Gralén (*1*) reported that the molecule of bovine catalase with the sedimentation constant of 11.3 *S* was split into smaller components or subunits when the enzyme solution was made up to pH 9.9. In the resultant solution, they identified two components with sedimentation constants of 1.2 *S* and 4.5 *S*. The implication of this interesting observation has, however, been left unelucidated until now. Considering the importance of the phenomenon in clarifying the nature of catalase molecule, we repeated the experiment of Sumner and Gralén, extending the range of pH applied from 7.0 to 12.0. By measuring simultaneously the sedimentation pattern and enzymatic activity of the solution, it was revealed that the product of alkali splitting of catalase molecule was, in disagreement with the results reported by Sumner and Gralén, homogeneous and showed a sedimentation constant of 3.1 *S*. The splitting of the molecule proceeded to completion at pH 12.0, where the enzyme activity also disappeared completely. The changes in sedimentation pattern and catalase activity were found to be partially reversible, giving rise to two components, one showing a sedimentation constant of 5.8–6.4 *S*, and the other having a a sedimentation constant similar to that of native catalase. Described herein are the experimental results and some deductions drawn from them.

EXPERIMENTAL

Catalase Preparation—Bovine liver catalase purified to crystalline state according to the method of Shirakawa (*2*). It was dissolved in buffer solution of various pH (1/10 mole phosphate buffer for pH 7.0 and 8.6, and 1/10 mole carbonate buffer for pH 9.9 and 11.0) or in a NaOH solution (for pH 12.0) and the solutions were subjected to various observations described below. The concentration of catalase was determined by the micro-Kjeldahl method.

Sedimentation Constant—Ultracentrifugal analyses were carried out at 59,780 r.p.m. (260, 000 × *g*) with a Spinco Ultracentrifuge, Model E. For the determination of molecular weight and axial ratio, the sedimentation constant at zero concentration was estimated by extrapolating the data at various concentrations. The percentage ratio of the split component to the total catalase dissolved was estimated from the peak areas in the sedimentation pattern in the concentration range of 3.25–8.65 g. of catalase dissolved per liter.

Diffusion Constant—Diffusion constants were measured by both maximum ordinate area method and second moment method (*3*, *4*) with a Hitachi diffusion apparatus equipped with a Philpot-Svensson type of Schlieren optical system (*5*, *6*).

Enzymatic Activity—Catalase activity was determined by the method of Euler and Josephson (*7*) under various conditions.

RESULTS AND DISCUSSION

The sedimentation and diffusion constants of the bovine liver catalase used were found to be: $S_{W.20}=11.2\ S$ and $D_{W.20}=4.1\times10^{-7}$ cm^2./sec. The molecular weight and axial ratio calculated from these values were 248,000 and 5.0, respectively, which agree with the values reported by Sumner and Gralén (*1*, *8*).

When the catalase was dissolved in alkaline solutions, two peaks appeared generally in the sedimentation pattern; one corresponding to the native catalase and the other having a sedimentation constant of 3.0–3.8 *S*. The small component was designated as component (I). The ratio, *f* (in per cent) of this component to the total catalase increased with the increase of alkalinity, as may be seen from the data presented in Table I. The table shows that more catalase was split with the increase of pH and the solution of pH 12.0 contained only the split molecules.

Table I

Formation of Component (I) by Alkali Denaturation

pH	Concentration of catalase used (*g./l.*)	Value of *f* (%) for component (I)
9.9	6.27	12
11.0	8.65	73
12.0	5.45	100

The relative catalase activity under various pH conditions is shown on the left column of Table III, in which the relative activity at pH 7.0 was taken as unity. At pH 12.0, where the ratio, *f* of component (I) was 100 per cent, no catalase activity was detected, indicating that component (I) was entirely deprived of the enzyme activity. It should, however, be noticed that between the activities and the values of *f* shown in Table I and III there seems to be no linear relationship. This is, probably, due to the situation that the activity of unsplit catalase molecule was not the same at different pH's.

The reversibility of the denaturation process was tested in the following way. Catalase was first dissolved in an alkaline solution of a fixed pH and then the pH of the solution was adjusted to 7.0 by adding 1/2 mole phosphate buffer. The sedimentation patterns of the solutions thus prepared indicated that, in the reverse process, component (I) disappeared completely and a new component having the sedimentation constant of 5.8–6.4 *S* appeared in addition to the component having the sedimentation constant similar to that of native catalase. The new component was designated as component (II) and the values of *f* for the component are listed in Table II.

TABLE II

Formation of Component (II) in the Reverse Process of Alkali Denaturation

pH change	Concentration of catalase used (*g./l.*)	Value of *f* (%) for component (II)
9.9–7.0	4.50	0
11.0–7.0	5.79	16
12.0–7.0	3.25	41

In parallel, the catalase activities of the neutral solution prepared in the same manner as above were measured (See Table III). From the results presented in Tables II and III, it may be seen that, when the enzyme was pretreated at pH 9.9 and later placed in a solution of pH 7.0, the form of catalase molecule as reflected in the sedimentation pattern recovered completely, but there was 14 per cent loss in the enzyme activity. With the increase of pH of the pretreatment, more component (II) was formed in the reverse process.

TABLE III

The Change in Catalase Activity by Alkali Denaturation and its Reversibility

(The relative activity at pH 7.0 was taken as unity)

pH	Relative activity (%)	pH change	Relative activity (%)
8.6	88	8.6–7.0	95
9.9	76	9.9–7.0	86
11.0	60	11.0–7.0	69
12.0	0	12.0–7.0	50

It is worth noticing that, when the enzyme pretreated at pH 12.0—where the enzyme activity disappeared completely—was returned to a solution of pH 7.0, there occurred 50 per cent recovery of the enzyme activity. This fact might be construed as indicating the larger component produced in the recovery process was capable of displaying enzyme activity. Whether or not component (II) had any catalase activity could not be ascertained from our experimental data.

With the enzyme solution treated at pH 12.0, in which all the enzyme molecules were split into component (I), the sedimentation and diffusion constants of the component at its zero concentration were determined to be: $S_{W.\,20}=3.1\ S$ and $D_{W.\,20}=3.3\times10^{-7}\ \mathrm{cm}^2/\mathrm{sec}$. The molecular weight and the axial ratio calculated from these values were 85,000 and 1.8, respectively. Striking is the fact that both the molecular weight and axial ratio of component (I) were approximately one third of those of native catalase. It may, therefore, be inferred that the catalase molecule is made up of three particles of component (I), which are connected lengthwise to form a rod-shaped molecule. Along the same line of reasoning, component (II) may be supposed to be a dimer of component (I), although we have as yet no data allowing to estimate the molecular weight of component (II).

It is known that the catalase molecule has four heme groups attached on the protein moiety. The question as to in what manner the four groups are distributed to the three molecules of component (I) is left open to further investigations.

SUMMARY

1. The process of alkali denaturation of bovine liver catalase was investigated by following the changes of sedimentation pattern and enzyme activity of the solutions under various pH conditions from 7.0 to 12.0. It was found that the catalase molecule is split by alkali into homogeneous components having a sedimentation constant of 3.1 *S*. The splitting proceeded to completion at pH 12.0, where also the enzyme activity disappeared completely.

2. The molecular weight and axial ratio of the split component as determined by the observation of sedimentation and diffusion constants were computed to be 85,000 and 1.8, respectively. Comparing these values to those of native catalase, it was deduced that the catalase molecule is composed of three particles of the 3.1 *S* component, connected lengthwise to form a rod-shaped molecule.

3. When the alkali-treated catalase solution was neutralized (to pH 7.0), the catalase activity recovered partially, and two peaks appeared in the sedimentation pattern. The sedimentation constant for one peak agreed with that of native catalase and the constant for the other peak suggested the existence of a dimer of the 3.1 *S* component.

The author wishes to express his sincere gratitude to Prof. K. Shibata and Dr. M. Nagahisa for their kind guidance and encouragement and also to Prof. H. Tamiya for his many suggestions and advices in preparing this manuscript.

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RIBONUCLEASE OF BACILLUS SUBTILIS

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The occurrence of microbial ribonuclease (RNase) has been reported by several workers (*1–4*), but most of the preparations reported so far except the RNase T_1 from Takadiastase by Sato and Egami (*5*) were not so much purified.

RNase of *Bacillus subtilis* was accumulated in a medium as well as amylase and protease. A 400 fold purification was achieved by acid treatment, ammonium sulfate fractionation and column chromatography. The results indicated that there were two types of RNases in a culture medium of *B. subtilis*.

The present communication deals with the separation, characterization and partial purification of RNase of *B. subtilis**.

METHODS

Yeast Nucleic Acid (*RNA*)—Commercially obtained yeast RNA purified as described by Frisch-Niggemeyer and Reddi (*6*) was used as substrate of RNase.

Medium—The composition of *Soy* medium was as follows: soluble starch 8 per cent, Na citrate 0.04 *M*, $(NH_4)_2HPO_4$ 0.15 *M*, KCl 0.02 *M*, $MgSO_4 \cdot 7H_2O$ 0.002 *M*, $CaCl_2$ 0.001 *M*, ethyl alcohol 1 per cent (*v/v*) and soybean extract. The latter was prepared by extracting 50 g. of soybean with about 250 ml. of 0.1 per cent NaOH by boiling for 1 hour. The entire extract was used per 1 liter of medium, pH=7.2.

Bacterial Strain –Unless otherwise stated *B. subtilis* H was used throughout these experiments.

Assay Procedure—RNase activity was determined as described by Frisch-Niggemeyer and Reddi (*6*) except that 1/15 *M* phosphate buffer pH 7.3 was used and that incubation was carried out at 37° for 15 minutes.

The activity corresponding to 1 μg. of crystalline pancreatic RNase incubated under the same experimental condition was defined as one enzyme unit.

Amylase was assayed by the method of Hagihara (*7*), protease by the method of Charney and Tomarelli (*8*), and protein content by the method of Lowry *et al.* (*9*).

RESULTS

Production of RNase by B. subtilis

Cultures were grown in a *Soy* medium with shaking at 30°. Aliquots were withdrawn and centrifuged at 10,000×*g* for 10 minutes. Cells were washed twice with 1/15 *M* phosphate buffer at pH 7.3 and lysed by the addition of 100 μg./ml. of lysozyme. RNase activities of the medium and of the lysed preparation were determined respectively. Growth rate of cells was determined by measuring the turbidity of washed cell suspension at 570 mμ. RNAase as well as amylase began to appear in the medium after the growth reached to a stationary phase as shown in Fig. 1.

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* A preliminary report of this paper has already been published in *Biochim. et Biophys. Acta*, **30**, 430 (1958)

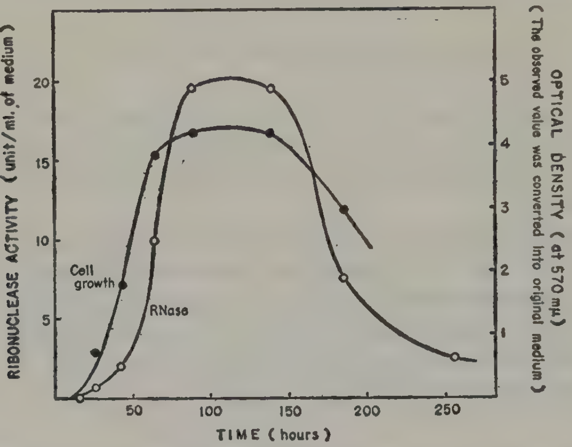

FIG. 1. Accumulation of RNase in a medium by *B. subtilis.*

RNase activity in the cells was less than 0.2 per cent of RNase activity in a medium all the while. It was not recognized that RNase within the cells increased remarkably in a particular growth phase. Therefore RNase is an exoenzyme as are amylase and protease in this organism. Perhaps RNase activity in lysed cell preparation was due to the contamination of a

TABLE I

RNase Production of B. subtilis

Each strain was grown in *Soy* medium under the same condition. After 60 hours the medium was separated by centrifugation, and RNase and amylase activities were determined.

Strain	RNase (*units/ml.*)	Amylase (*units/ml.*)
B. subtilis B-4-4	1.3	∧
B-4-1	0.12	∧
B-4-2	3.3	1.5
B-4-3	2.9	4
H	6.4	200
N	∨	750
K	∨	2,000

culture medium which was not completely washed out.

As shown in Table I, the capacity of RNase formation was distributed evenly in various strains although the ability of amylase formation was very different from strain to strain. The capacity of RNase formation by this organism depends on the compositions of a medium.

Isolation of RNase

The medium (1.9 liter) was collected by centrifugation at 7,000×*g* for 10 minutes after the cells were grown in *Soy* medium for 70 hours. As the supernatant was slightly turbid, it was filtered with the aid of Hyflo Super Cel. To this clear filtrate, 100 ml. of 4 *N* H_2SO_4 was added. After centrifugation the residue was discarded and the supernatant was brought to 0.57 saturation by the addition of solid ammonium sulfate. RNase was precipitated together with a large amount of polysaccharide-like substances. After the addition of Hyflo Super Cel, the precipitate was collected by filtration and the filter cake was extracted with 250 ml. of distilled water. To this extract solid ammonium sulfate was added to 0.15 saturation and the precipitate produced was collected by centrifugation. The precipitate was dissolved in 250 ml. of distilled water, and brought to 0.48 saturation by the addition of solid ammonium sulfate. The precipitate was discarded and clear supernatant was brought to 0.61 saturation. The precipitate which no longer contained polysaccharide-like substance was separated by centrifugation, dissolved in 2.5 ml. of distilled water and dialysed for 6 hours against distilled water.

At this stage RNase was purified about 45 fold with the yield of about 10 per cent. An outline of purification procedure is summarized in Table II.

TABLE II

Purification of RNase of B. subtilis

Specific activity was calculated as the ratio of unit of RNase/ml. to tyrosine content/ml. In case of steps 1 and 2, tyrosine content as determined with the dialyzed samples.

	Volume (*ml.*)	RNase units/*ml.*	Total unit	Specific activity	Yield (%)
1. Culture medium	1,900	10.5	20,000	0.020	100
2. Supernatant after removal of precipitate by the addition of H_2SO_4	2,000	10.0	20,000	0.056	100
3. Precipitate at 0.57 $(NH_4)_2SO_4$	250	41.0	11,000	0.17	52
4. Precipitate at 0.15 $(NH_4)_2SO_4$	250	16.0	4,000	—	19
5. Precipitate between 0.48 and 0.61 $(NH_4)_2SO_4$ saturation	2.5	850	2,100	0.86	10

Properties

pH Optimum—For the determination of the RNase activity at different pH values, four different buffers were used. The pH optimum in these buffers was found to be at 7.5 as shown in Fig. 2.

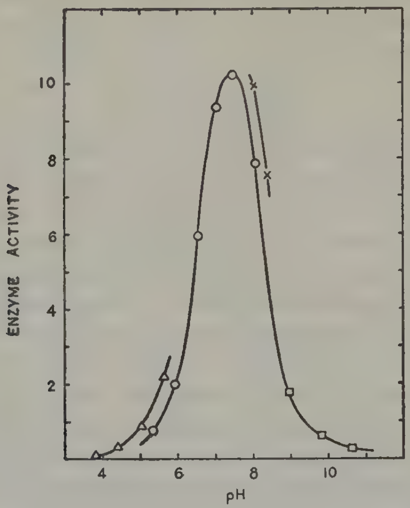

FIG. 2. Influence of pH on enzyme activity.
The final concentration of buffer was 1/15 *M*. —△—; Acetate buffer, —○—; Phosphate buffer, —×—; Tris buffer, —□—; Carbonate-bicarbonate buffer.

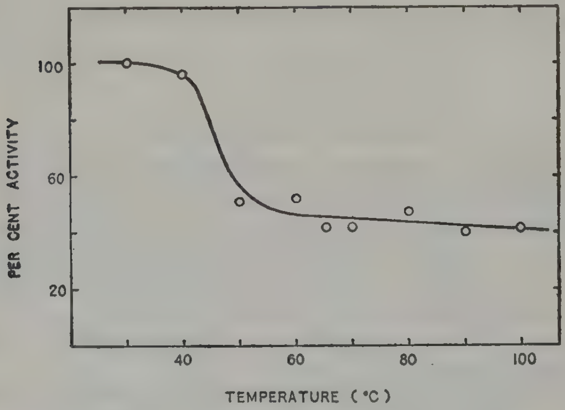

FIG. 3. Effect of temperature on stability of RNase.
The enzyme preparation was diluted to 0.1 units/ml. suitable for enzyme assay before the heat treatment.

Stability—Aqueous solution of RNase is quite stable over a wide range pH when kept at temperatures below 5°. Heat stability was tested by heating samples of the enzyme at pH 7.3 for 10 minutes in a water bath at temperatures ranging from 30° to 100°. At 45° the activity dropped sharply to about 60 per cent of the original activity, but at 100° about 40 per cent was still retained (Fig. 3). Heat stability of enzyme depended on enzyme concentration. Concentrated preparation was less susceptible to inactivation by heat treatment.

Impurities—To test the influence of hydrogen ion concentration on the stability of RNase, samples were incubated at 25° for 18 hours in 0.1 *M* buffer at pH ranging from 3.8 to 10.6. It was shown that RNase was most labile at pH 7. This might be explained by the assumption that partially purified RNase preparation contained protease produced by the organism. But actually no protease activity nor amylase activity could be detected under the conditions described in the experimental part, indicating that each of the impurities, if present, was less than 0.1 per cent. The determinations of deoxyribonuclease, phosphomonoesterase and phosphodiesterase activity were not attempted.

Column Chromatography of RNase

Further purification of RNase by the use of calcium phosphate gel and alumina cγ gel treatment was unsuccessful.

Purification using Amberite IRC-50 (XE-64) as described by Hirs (*10*) was performed.

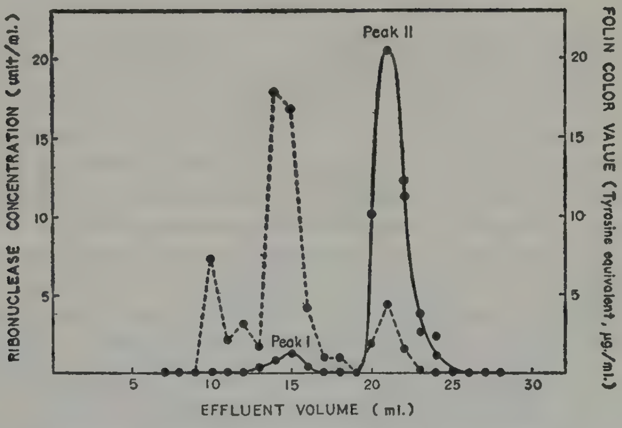

FIG. 4. Chromatography of RNase of *B. subtilis.*

Elution was performed with 0.2 *M* sodium phosphate buffer at pH 5.91 from a column (0.9 × 20 cm.) of Amberite IRC-50 (XE-64) equilibrated with the same buffer. The solid line represents RNase activity, and the broken line protein content. The flow rate was about 1 ml./cm.^{2}/hour.

As shown in Fig. 4, it was indicated that this preparation consists of at least two types of RNases and one of them (Peak 11) was clearly separated from a contaminated protein. RNase contained in effluent fractions from No. 20 to No. 24 was purified about 400 fold and the specific activity of this enzyme was comparable to that of crystalline pancreatic RNase.

Heat stability test suggested that the two enzymes differed as to stability to heat, but per cent activity of each enzyme after heat treatment (100°) was the same under the experimental conditions as described under Fig. 3.

DISCUSSION

Hitherto a number of exoenzyme of *B. subtilis* was discovered and some of them, for example amylases and proteases, were already crystallized (*11–14*). But RNase had not attracted investigator's attention so far.

Nomura, Hosoda and Nishimura reported that the lysozyme-lysed preparation of *B. subtilis* was capable of synthesizing amylase almost comparable in magnitude to the intact cells (*15*, *16*). In this system, it was necessary to add polyvinylsulphate which was supposed to inhibit RNase action. At first the lability of the system was presumed to be due to the presence of intracellular RNase which was activated in the cource of lysis in analogy with the observations of Kozloff (*1*). Singularly, however, RNase was found to be an exoenzyme and the content of intracellular RNase was negligibly small as compared with an exo-RNase.

The production of RNase was also activated by *Factor* which stimulated the formation of amylase and protease by the same organism (*17*).

Recently many investigators used RNase as a tool to prove that nucleic acid plays a role in protein synthesis. For example, enzyme formation, amino acid incorporation, cell growth, *etc.* were inhibited by the addition of pancreatic RNase to the system. But there may be a possibility that RNase might act in synthetic process of cells. RNA might be synthesized in the cells by the action of RNase in analogy to the observation by Naoi, Asano and Egami (*18*), who reported that polyguanylic acid was synthesized from guanosine 2',3'-cyclic phosphate by the action of RNase T_1, or RNase might play a role in supplying nucleotides to the cells which might reuse them for RNA synthesis. Isolation of highly purified bacterial RNase will provide a method to investigate the action of RNase within bacterial cell.

Microbial RNases reported so far except the RNase T_1 from Takadiastase were not so much purified as the one reported here as judged from the specific activities. Specific activity of RNase of Peak II was comparable to that of crystalline pancreatic RNase. Of interest was that *B. subtilis* produced two types of RNase and that chromatographic pattern of RNase was very similar to that of pancreatic RNases in connection with the observation by Hakin (*19*) who reported combined action of the pancreatic RNase A and B. RNases of *B. subtilis* were not adsorbed on a column which was equilibrated with 0.2 *M* sodium phosphate buffer (pH 6.47) used for the purification of pancreatic RNases. Therefore they appear to be slightly more acidic than pancreatic RNases.

The purification using a large scale column and an investigation of other properties of each enzyme are in progress.

SUMMARY

1. RNase of *B. subtilis* was accumulated in the culture medium as an exoenzyme. RNase production varied markedly with conditions of culture, phases of growth and strains.

2. Two types of RNases of *B. subtilis* were separated by column chromatography using the carboxylic acid cation exchange resin IRC-50 (XE-64).

3. By employing the acid treatment, ammonium sulfate fractionation and column chromatography, one of the enzymes was purified almost 400 fold. Its specific activity was comparable to that of crystalline pancreatic RNase.

4. Properties of RNase were reported,

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SOME MEASUREMENTS OF DISSOCIATION CONSTANTS OF URIC ACID AND CREATININE

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(Received for publication, July 26, 1958)

In studies of buffers in urine, the authors were under necessity to cite dissociation constants of urine buffers from literatures and found that many problems are left unsettled in determination of these constants.

Young and Musgrave (*1*) repoted the following value as the first dissociation constant of uric acid, $K_1=8\times10^{-6}$, while Bernouilli and Loebenstein's value (*2*) is $K_1=4.1\times10^{-6}$. With regard to the dissociation constant of creatinine, values of previous authors are presented in Table I. These values seem to coincide with one another in the scope of experimental error. However, effects of ionic strength of the solution upon the dissociation constants are not yet studied. Therefore it is difficult to utilize these values freely in any sort of solution of known ionic strength. In case of uric acid, it is also important to know the effect of formaldehyde, because formalin is often employed to dissolve uric acid.

TABLE I

Dissociation Constant of Creatinine

(Cited from Cannan and Shore's paper (1928)).

Authors	*M*	Temp.	pK_w-pK_b'	K_b
Mc. Nally (1926)	—	40.0	4.42	1.01×10^{-9}
Cannan & Shore (1928)	0.1	30.0	4.77	0.98×10^{-9}
,,	0.02	30.0	4.72	0.15×10^{-9}
,,	0.1	25.0	4.78	0.76×10^{-9}
Mc. Nally (1926)	—	25.0	4.71	0.70×10^{-9}
Eadie & Hunter (1926)	0.1	20.0	4.87	0.64×10^{-9}
Hahn & Barken (1920)	0.04	17.0	4.44	0.19×10^{-9}
Cannan & Shore (1928)	0.02	15.0	4.91	0.47×10^{-9}

In the present study, the authors attempted to determine the thermodynamic dissociation constant of uric acid as well as of creatinine, and to clarify effects of ionic strength and of formaldehyde upon them, so as to be able to calculate the apparent dissociation constant in any sort of test solution.

METHODS

Principles of measurement of dissociation constants of uric acid and creatinine are as follows. Dilute solutions of uric acid or creatinine were prepared by dissolving a certain amount of sample in water or NaCl solutions of from 0.05 *M* to 1.6 *M* as described below. 5 ml. of each solution was introduced into a glass electrode for titration, which was devised by the authors (see Fig. 1). Uric acid solution was titrated with 0.02 *N* NaOH, while creatinine solution with 0.02 *N* HCl. The temperature of the solution was 37°. The titration curve thus obtained was analysed according to Shima's principle (*3*), and the dissociation constant was calculated. The principle will be explained by utilizing titration curve of uric acid in Fig. 2.

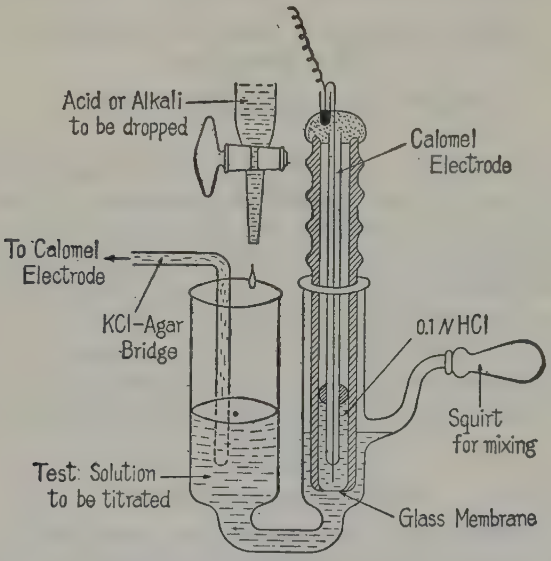

FIG. 1. A new type glass electrode for titration. Calomel electrode was prepared by electroplating Pt wire with Hg which was dipped in saturated calomel solution.

Put a point X on a titration curve, and take a point on the curve at a distance of *h* pH below (A) or above (B) the point X. Let *a* and *b* be differences of ml. of NaOH added to the solution up to points A and B respectively from that of the point X. The dissociation constant of weak acid can be calculated from the following formula, so far as Henderson-Hasselbalch's equation is applicable,

$$\mathrm{pH}=\mathrm{pK_a}'+\log\frac{10^h a-b}{10^h b-a} \quad \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots (1)$$

where $\mathrm{pK_a}'$ represents the apparent dissociation exponent of weak acid. For the weak base, the same formula is applicable if $\mathrm{pK_a}'$ is substituted with $\mathrm{pK_w}-\mathrm{pK_b}'$. $\mathrm{pK_b}'$ is the apparent dissociation exponent of weak base and $\mathrm{pK_w}$ is the dissociation exponent of water. From the measurements of $\mathrm{pK_a}'$ or $\mathrm{pK_w}-\mathrm{pK_b}'$ in solutions of various salt concentrations, effects of ionic strength upon the dissociation constant are derived and the thermodynamic constant was calculated.

Preparation of Solutions—6 mM uric acid was weighed and dissolved in 1 liter flask which contains a dilute solution of lithium carbonate of 0.6 g.

After 4 ml. formalin was added to the solution in order to prevent the precipitation of uric acid, the solution was acidified with 3 ml. of 6 N H_2SO_4, and the solution volume was finally adjusted accurately to 1 liter by adding distilled water. Thus the solution contains 6 mM uric acid, 0.6 g. $LiCO_3$, 9 mM H_2SO_4 and 1.6 g. formaldehyde, as formalin contains 40 per cent formaldehyde.

After the solution was evacuated completely by vacuum pump to eliminate CO_2, it was used as a titrable sample. A part of the solution was diluted in four fold with distilled water, and the diluted sample which contains 1.5 mM uric acid and 0.04 per cent formaldehyde was prepared. In this diluted sample, NaCl was added in proportion of 0.05, 0.1, 0.4 and 1.0 mole to liter solution respectively.

Creatinine was dissolved in water in a concentration of 100 mg./dl., and the solution was mixed half to half with sodium chloride solution of which concentration ranged from 0.2 to 3.2 M. Thus 50 mg./dl. creatinine was prepared in which NaCl was contained in concentration ranging from 0.1 to 1.6 M.

EXPERIMENTS AND DISCUSSIONS

1. The Dissociation Constant of Uric Acid—As the uric acid solution

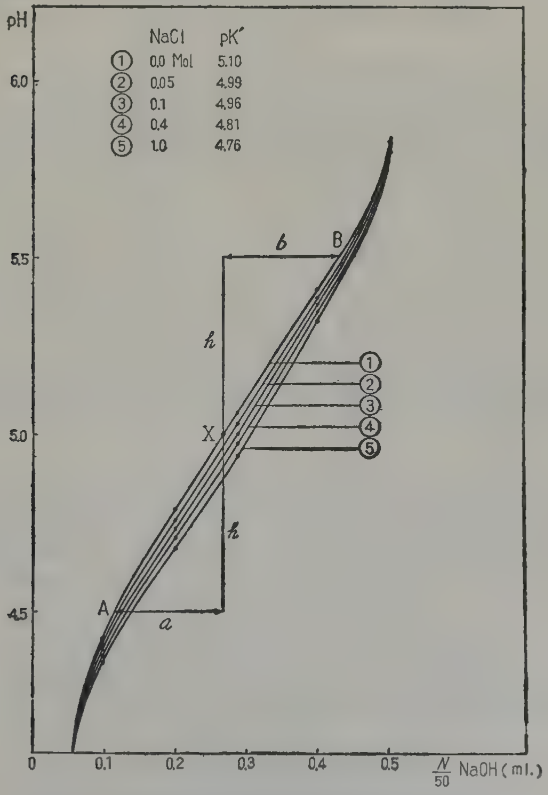

FIG. 2. Titration curves and pK′ of uric acid.
Uric acid 1.5 mM/liter, formaldehyde 0.04 per cent.

contained formalin for the prevention of precipitation, effects of formalin upon the dissociation constant of uric acid was at first examined. The solutions examined were 6 mM uric acid solution which contain 0.6 g. $LiCO_3$ per liter and various amounts of formalin (40 per cent) ranging from 0.2 to 2.0 ml. was added to each 100 ml. sample. The formaldhyde concentration in these solutions ranged from 0.026 to 0.260 *M*.

The solutions thus prepared were titrated with 0.02 *N* NaOH solution, and the titration curves were drawn visually by connecting the measured points so as to fit closely the theoretical curves which can be traced by aid of the equation (1).

The curve were analysed as described above. The value of *h* in the equation (1) was chosen to be 0.5 around the middle point of each curve (see Fig. 2), because the uric acid concentration is faily low and the fitness to Henderson-Hasselbalch's equation becomes worse at the end part of the titration curve. The apparent dissociation exponent pK′ thus calculated by the equation (1) corresponds to the first dissociation exponent and is presented with the amount of formaldehyde contained in the solution in Table II. It is shown that pK′ decreases as the amount of formalin increases. As uric acid is an imino acid, the fact may be explained by Levy's theory (*4*). According to it, amino acid as well as imino acid can combine with formaldehyde and exert influences on dissociations of these acids. For example, let R_1–NH–R_2–COOH be an imino acid, following two equilibriums may exist in the solution if formalin is added,

TABLE II

Effect of Formaldehyde on pK′ of Uric Acid

No. of solution	Formalin contained in 100 ml. solution	Formaldehyde concentration (*M*)	pK′ of uric acid
1	0.2 *ml.*	0.026	4.97
2	0.3 „	0.039	4.91
3	0.5 „	0.065	4.86
4	1.3 „	0.169	4.69
5	2.0 „	0.260	4.73

Uric acid was dissolved in Li_2CO_3 solution so that 6 mM was contained in 1 liter, and was partly neutralized with small amount of concentrated H_2SO_4. Its ionic strength, μ, was 0.04.

$$R_1NH_2^+R_2COO^- \rightleftarrows R_1NHR_2COO^- + H^+$$
$$R_1NHR_2COO^- + CH_2O \rightleftarrows R_1N(CH_2OH)R_2COO^-$$

Applying the mass law to these reactions,

$$\frac{[H^+][R_1NHR_2COO^-]}{[R_1NH_2^+R_2COO^-]} = K'$$
$$\frac{[R_1N(CH_2OH)R_2COO^-]}{[R_1NHR_2COO^-][CH_2O]} = L$$

are obtained. K′ and L are the constants of equilibriums. The ratio of total anion concentration to total imino acid being represented as α, the Henderson-Hasselbalch's equation may be derived as follows,

$$\mathrm{pH}=\mathrm{pK}'-\log(1+\mathrm{LF})+\log\frac{\alpha}{1-\alpha}\tag{2}$$

F represents the molar concentration of formaldehyde which remains uncombined. The value may be taken as the same with the total formaldehyde concentration and is constant, if formalin is added far excessively to imino acid. Accordingly, (2) may be written as follows,

$$\mathrm{pH}=\mathrm{pH_F}'+\log\frac{\alpha}{1-\alpha},$$

$$\mathrm{pK_F}'=\mathrm{pK}'-\log(1+\mathrm{LF})=-\log(\mathrm{K}'+\mathrm{LFK}')\tag{3}$$

Thus $\mathrm{pK_F}'$ is a function of formaldehyde concentration, and decreases as the amount of formalin added is increased.

From the results in Table II, the constants L and pK′ in the equation (3) can be calculated by the method of least square, as follows.

$$\mathrm{pK_F}'=5.08-\log(1+8.198\mathrm{F})\tag{4}$$

5.08 corresponds to pK′ of 6mM uric acid solution which contains no formaldehyde.

To clarify effects of ionic strength on the dissociation constant, the uric acid solution of 6mM was diluted into four folds with water or NaCl solutions of various concentrations, and 1.5mM uric acid solutions which contained 0.04 per cent (0.013 *M*) formaldehyde, and various amounts of NaCl ranging from 0.00 to 1.0 *M* were prepared as described above. Titrating these solutions with 0.02 *N* NaOH as previously, apparent dissociation constants were calculated. Fig. 2 presents the titration curves thus obtained. It is clearly shown that the level of curve is lowered by addition of NaCl, as the dissociation constant is influenced by ionic strength. The dissociation exponents, pK′, calculated by the equation (1) are plotted in Fig. 3, in which the ionic strength of the solutions is represented with μ. According to Debye and Hückel, pK′ can be calculated by the following limiting equation (*5*),

$$\mathrm{pK}'=\mathrm{pK}-0.5\sqrt{\mu}+\mathrm{B}\mu.$$

The constants, pK, and B in the equation were calculated by the method of least square from the experimental data, and the following empirical formula was obtained,

$$\mathrm{pK}'=5.13-0.5\sqrt{\mu}+0.082\,\mu\tag{5}$$

From this equation, the dissociation exponent of the solution of zero ionic strength is 5.13. As the solution contains 0.013 *M* formaldehyde, this value includes some effect of formaldehyde which is represented by the term, log (1+8.198 F) in the equation (4).

As this correction term corresponds to 0.0443 in this case, pK is corrected as to be 5.17 (=5.13+0.04). This value corresponds to the thermodynamic dissociation exponent which is effected neither by ionic strength nor by formaldehyde.

Another mean to obtain the thermodynamic dissociation exponent is to correct pK′ of 6 mM uric acid solution without formalin stated above by aid of the equation (5). From the equation (4), pK′ of 6mM uric acid without formalin equals 5.08. As the ionic strength in this solution is about 0.03 *M*, the correction term for it, $(0.5\sqrt{\mu}-0.082\,\mu)$, is 0.08.

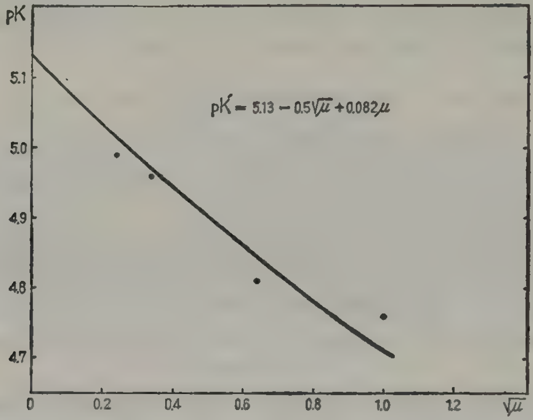

FIG. 3. Effect of ionic strength upon the dissociation constant of uric acid. Uric acid 1.5 mM/liter, Formaldehyde 0.04 per cent.

Thus the thermodyamic dissociation exponent is 5.16 (=5.08+0.08), which coincides with the value calculated above. The mean of the thermodynamic dissociation exponent of uric acid obtained by two means is 5.165, which may be useful to calculate pK′ in routine solutions of uric acid. Effects of ionic strength may be estimated from the equation (5) so far as it is below $\mu=1.0$ and effects of formalin from the equation (4).

2. *The Dissociation Constant of Creatinine*—Creatinine is an amphoretic electrolyte, and is believed to dissociate as an anion in acid side, while it dissociates into cation in alkaline side. It means that creatinine acts as a base in acid solution according to the classical theory of amino acid dissociation, while it is an acid in alkaline solution. To discuss the dissociation in urine, the base dissociation is important, and is expressed by the following Herderson-Hasselbalch's equation,

$$\mathrm{pH}=\mathrm{pK_w}-\mathrm{pK_b}'-\log\frac{\alpha}{1-\alpha},$$

where α is the degree of dissociation.

In Table III, there are presented values of $(\mathrm{pK_W}-\mathrm{pK_b}')$ which were determined by Shima's formula (*3*) from titration curves of creatinine solutions with 0.02 *M* HCl. Solutions contain various amounts of NaCl and have various ionic strength, μ. By utilizing Debye-Hückel's limiting equation, the empirical formula between $(\mathrm{pK_W}-\mathrm{pK_b}')$ and ionic strength was derived as follows,

$$(pK_w - pK_b') = 4.75 + 0.5\sqrt{\mu} - 0.43\mu \quad \ldots\ldots\ldots\ldots\ldots\ldots\ldots (6)$$

The constants of the formula were calculated by he dmetod of least square from data presented in Table III. $(pK_W - pK_b')$ at zero ionic strength is 4.75, and thus the thermodynamic dissociasion exponent of creatinine, pK_b, is calculated to be 8.76 by adopting 23.505 as pK_W at 37° according to Michaelis (*6*).

Cannan and Shore (*7*) determined $(pK_W - pK_b')$ of creatinine solution by the hydrogen gas electrode, and obtained 4.72 at 30°, which coincides well with the authors'. The formula may be applied to any dilute solution of known ionic strength so far as it is below 0.4 *M*.

TABLE III

($pK_w - pK_b'$) of Creatinine Solution (4.4 mM/liter)

No.	Conc. of NaCl (*M*)	Ionic strength (μ)	$pK_w - pK_b'$
1	0.0	0.0044	4.80
2	0.1	0.1044	4.86
3	0.2	0.2044	4.87
4	0.4	0.4044	4.90
5	1.0	1.0044	4.92
6	1.6	1.6044	4.93

SUMMARY

Dissociation exponents of uric acid and creatinine (at 37°) were estimated by aid of Shima's formula, and following results were obtained.

1. Effects of formaldehyde upon the first acid dissociation exponent of uric acid in 6 mM solution, pK_F', was derived from Levy's equation, and the following formula was obtained.

$$pK_F' = 5.08 - \log(1 + 8.198F),$$

where F is molar concentration of formaldehyde (below 0.26 M).

2. With a series of uric acid solutions of 1.5 mM, in which 0.04 per cent formaldehyde and various amounts of NaCl up to 1 *M* were contained, effects of ionic strength, μ, upon the dissociation exponent, pK′, were examined, and the following equation was obtained.

$$pK' = 5.13 - 0.5\sqrt{\mu} + 0.082\mu$$

3. From the above two equations, the thermodynamic dissociation exponent of uric acid which has neither formalin effect nor salt effect is calculated to be 5.165 at 37°.

4. The base dissociation exponent pK_b' of creatinine in solutions of various ionic strength (below 0.4 *M*) is expressed by the following equation.

$$pK_w - pK_b' = 4.75 + 0.5\sqrt{\mu} - 0.43\mu,$$

where pK_W is the dissociation exponent of water. The thermodynamic dissociation exponent of creatinine is thus calculated to be 8.76 at 37°.

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CHEMICAL MODIFICATION ON TAKA-AMYLASE A

I. DINITROPHENYLATION OF TAKA-AMYLASE A

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The chemical structure of Taka-amylase A (TAA) has been the object of an extensive investigation in our laboratory during the past years, and its amino acid composition (*1*), carbohydrate components (*2*), N-terminal amino acid (*3*), and C-terminal groups (*4*) have already been elucidated. However, no information has as yet been available concerning the relationship between the chemial structure of TAA and its enzymic activity, although studies along these lines have already been made on some of the other enzymes.

Little and Caldwell (*5*) have reported that amino groups, but not phenolic hydroxyl groups, of pancreatic α-amylase are essential for its enzymic activity. Myrbäck and Frostell (*6*) have found that amino groups of malt α-amylase participate in the combination of the enzyme with its substrate. Recently, Yamamoto (*7*) suggested that tyrosyl and/or tryptophanyl groups may be involved in the activity of a bacterial α-amylase.

The present investigation was undertaken to obtain information concerning the essential groups of TAA by modifying the enzyme protein with chemical reagents which are known to react with definite groups of proteins. Fluorodinitrobenzene (FDNB) and dinitrobenzene sulfonate (DNBS) were chosen as the reagents because by these reagents the number of reagents combined could be relatively easily determined. FDNB is known to react with sulfhydryl, phenolic hydroxyl, imidazolyl and amino groups of proteins in slightly alkaline solutions (*8*) and has been extensively used for the determination of N-terminal groups of a number of proteins. DNBS, on the other hand, has been shown by Eisen *et al.* (*9*) to react specifically with ε-amino groups of lysine residues. The actions of the both reagents result in the dinitrophenylation of the susceptible groups.

The results of the experiments to be reported in the present paper seems to suggest that ε-amino groups of lysine residues are not essential for the amylase activity, but some special phenol groups of tyrosine residues are more closely connected to the emergence of the enzymic activity of TAA.

EXPERIMENTAL

Taka-amylase A (*TAA*)—Crystalline TAA was prepared from "Takadiastase Sankyo" according to the method described in a previous paper (*10*) and recrystallized three times from aqueous acetone. The concentrations of TAA were estimated from nitrogen contents determined by the micro Kjeldahl method. The average molecular weight of 53,000 (*3*) was employed for TAA in calculating its molar concentration and the number of reagent molecule combined with one molecule of the protein. Amylase activity was measured at pH 5.3 (acetate buffer), 37° by determining its saccharogenic activity by the method of Fuwa using amylose as substrate (*11*).

Dinitrophenylation of TAA with FDNB—A 0.94×10^{-4} *M* solution of TAA was treated with FDNB in the presence of 0.1 *M* veronal buffer at 37°; the molar ratio being 50 moles of FDNB per mole of TAA After desired periods of reaction time, the amount and the kind of amino acid residues combined with the reagent were determined by the following method. DNP-TAA was precipitated by the addition of an equal volume of 20 per cent trichloroacetic acid, separated from the supernatant by centrifugation and washed with acetone four times. The washed DNP-TAA was dried in a desiccator and hydrolyzed with 6 *N* hydrochloric acid in a sealed tube for 20 hours at about 110°. The hydrolysate was extracted with distilled ether and the aqueous layer was evaporated *in vacuo*. The latter was used to determine the quantities of ε-DNP-lysine and *O*-DNP-tyrosine by the column chromatography as described in the following section.

Ion Exchange Chromatography of ε-DNP-Lysine and O-DNP-Tyrosine—Chromatographic determination of ε-DNP-lysine and *O*-DNP-tyrosine was carried out by a modification of Seki's method (*12*) using a 0.9 × 15 cm. column of Amberlite IR-112. The solvent system employed was a mixture of 3 volumes of 2.5 *N* hydrochloric acid and 1 volume of acetic acid. The sample dissolved in 1–2ml. of the solvent was applied to the column and then eluted at a rate of 6–8 ml. per hour. Each 1.5 ml. of the effluent was collected with a fraction collector. The concentration of *O*-DNP-tyrosine and ε-DNP-lysine in each fraction was determined photometrically at 300 mμ and 360 mμ, respectively. As shown in Fig. 1,

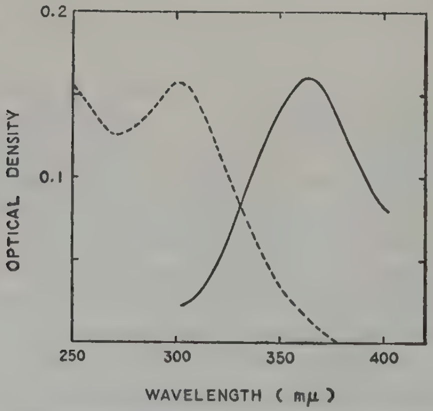

FIG. 1. Absorption spectra of ε-DNP-lysine and *O*-DNP-tyrosine in 2.5 *N* HCl-CH_3COOH (3 ; 1).
——: ε-DNP-lysine 1.0×10^{-5} *M*, ----: *O*-DNP-tyrosine 1.5×10^{-5} *M*.

ε-DNP-lysine has an absorption maximum at 360 mμ and *O*-DNP-tyrosine at 300 mμ. Such a difference of the absorption maxima permitted the precise determination of the both compounds even in their mixture. The recovery of ε-DNP-lysine during acid hydrolysis was 90–95 per cent as described in a previous paper (*3*) and that of *O*-DNP-tyrosine was found to be 95 per cent. Fig. 2 shows a typical elution curve showing the separation of ε-DNP-lysine and *O*-DNP-tyrosine.

Dinitrophenylation of TAA with DNBS—A 0.5×10^{-4} *M* solution of TAA was treated at 25° for various intervals with DNBS at a molar ratio of 1,000 moles of DNBS per mole of TAA in the presence of triethylamine-hydrochloric acid buffer (pH 10.7) and 0.05 *M* calcium acetate. The amount of ε-DNP-lysine in the resulting DNP-TAA was determined as follows; DNP-TAA was precipitated by the addition of an equal volume of 20 per cent trichloroacetic acid and washed with ethanol to remove the excess of DNBS. DNP-TAA thus obtained was dissolved in 10 *N* hydrochloric acid and the concentration of ε-DNP-lysine was determined photometrically at 360 mμ.

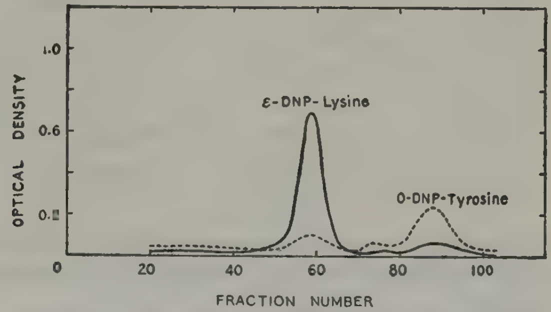

FIG. 2. Elution curve of ε-DNP-lysine and *O*-DNP-tyrosine. Sample was the hydrolysate of 0.42×10^{-6} mole DNP-TAA after dinitrophenylation with FDNB (50 molar ratio to 1 mole of TAA) in the medium of pH 8.6 at 37° for 120 minutes.
——: measured at 360 mμ, ----: measured at 300 mμ.

RESULTS AND DISCUSSION

Fig. 3 shows the effect of dinitrophenylation at 37° with FDNB on the enzymatic activity of TAA. It will be seen that only a very low inactivation of the enzyme was observed when the reaction was performed at pH 6.0 or 7.0. It was in fact found that TAA was practically not dinitrophenylated under these conditions. When, however, the pH of the reaction medium was increased, the inactivation proceeded more rapidly. At pH 9.0 the activity was completely abolished after 3 hours. When the reaction temperature was lowered to 0°, however, the inactivation rate was very slow even at pH 9.0 and 80 per cent of the original activity was still left after 4 hours incubation under these conditions.

In Fig. 4 are illustrated the results of an experiment in which TAA was dinitrophenylated with FDNB at pH 8.6 and the decrease in the amylase activity was followed in relation to the number of lysine and tyrosine residues dinitrophenylated. The inactivation of the enzyme progressed as the number of *O*-DNP-tyrosine and ε-DNP-lysine residues increased. The activity seems to be completely lost when 2 tyrosine and 5 lysine residues in the protein are dinitrophenylated. It was, however, found that the N-terminal alanine residues of the enzyme molecule was not yet dinitrophenylated even at the complete loss of the activity. These experimental data suggest that some

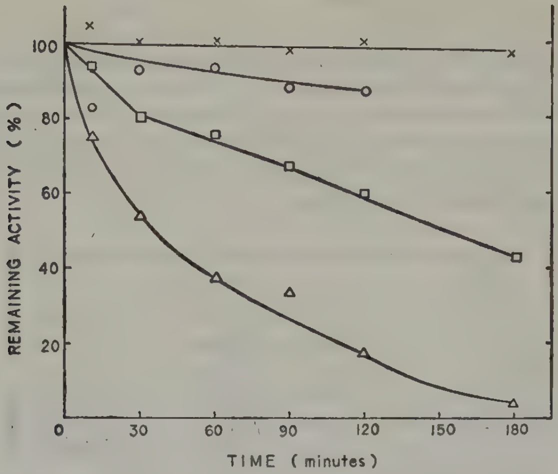

FIG. 3. Change of amylase activity during dinitrophenylation of TAA with FDNB (50 molar ratio to 1 mole of TAA) in the media of various pH at 37°.

—○— in pH 6.0, —×— in pH 7.0, —□— in pH 8.0, —△— in pH 9.0.

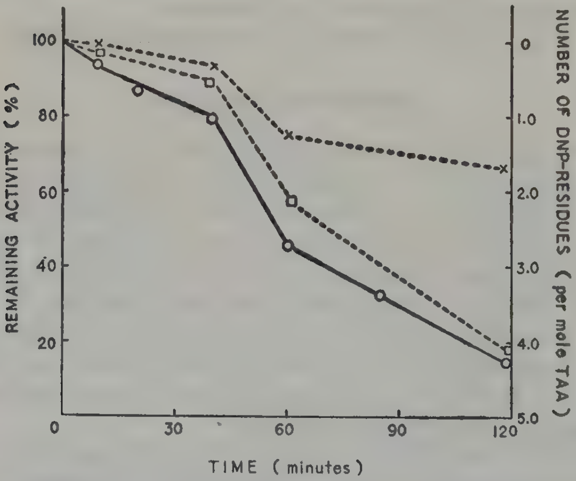

FIG. 4. Change of amylase activity and number of combined DNP-residues during dinitrophenylation of TAA with FDNB (50 molar ratio to 1 mole TAA) in the medium of pH 8.6 at 37°.

—○— amylase activity, --×-- number of *O*-DNP-tyrosine, --□-- number of ε-DNP-lysine.

of lysine and/or tyrosine residues are indispensable for the activity of TAA.

DNBS is known to attack specifically the ε-amino group of lysine residue. This reagent was, therefore, reacted with TAA in order to obtain further information of the essential groups of the enzyme. The reaction conditions described in the experimental section were chosen on the basis of the following preliminary observations. The inactivation of TAA was extremely slow when the dinitrophenylation was carried out below pH 10 or at a molar ratio of 100 moles of the reagent per mole of the enzyme. When the molar ratio was raised to 1,000 moles of DNBS per mole of TAA and the pH was fixed at 10.7, the reaction velocity became measurable although it was still rather slow. Calcium acetate was, therefore, added to the reaction mixture to avoid possible denaturation of the enzyme which might occur during prolonged incubation at the alkaline pH.

That only lysine residues of the protein are dinitrophenylated under these conditions was confirmed by comparison of absorption spectra of dinitrophenylated samples of TAA and that of ε-DNP-lysine. Fig. 5 shows the absorption spectrum in 10 N hydrochloric acid of ε-DNP-lysine together with those of TAA samples dinitrophenylated with FDNB and DNBS. The absorption curves for the DNP-proteins were drawn by subtracting the absorption due to the original protein from those of the DNP-proteins. It will be seen that the curve for the DNBS-dinitrophenylated TAA is identical with that for ε-DNP-lysine, and that the curve for the FDNB-dinitrophenylated TAA exhibits an extra absorption at 300 mμ owing to the dinitrophenylation of tyrosine.

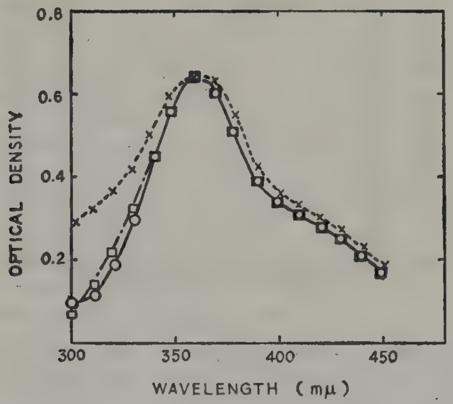

FIG. 5. Absorption spectra in 10 N HCl of ε-DNP-lysine and DNP-TAAs obtained by the dinitrophenylation with FDNB and DNBS. The absorption of TAA was subtracted from that of each DNP-TAA.

—○— ε-DNP-lysine, 4×10^{-5} M in 10 N HCl, --×-- DNP-TAA dinitrophenylated with FDNB, •—□•— DNP-TAA dinitrophenylated with DNBS.

Fig. 6, shows the decrease in the enzymatic activity as well as the number of lysine residues dinitrophenylated during reaction of TAA with DNBS. As can be seen from the figure, 11 out of the total 22 lysine residues in TAA were dinitrophenylated after 50 hours incubation under the condition employed. It was surprising to find that the enzyme still retained about 60 per cent of the original activity even after such a rather drastic treatment. It seems, therefore, very likely from these results that the lysine residues of TAA, at least those 11 which were dinitrophenylated in the above experiment, are not directly concerned in the activity and the decrease of the enzymic activity is actually due to a certain of the modification of the secondary structure of the enzyme molecule by dinitrophenylation of lysine residues.

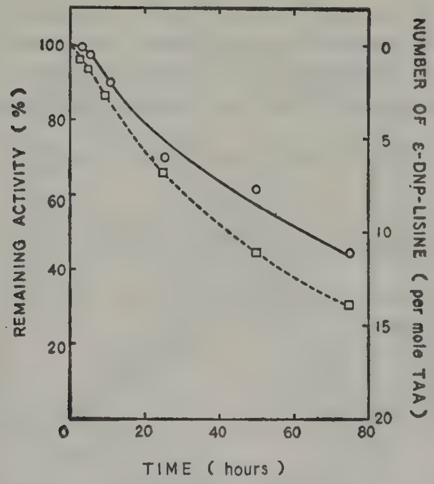

FIG. 6. Change of amylase activity and number of ε-DNP-lysine during dinitrophenylation of TAA with DNBS (1,000 molar ratio to 1 mole of TAA) in the presence of triethylamine—HCl buffer (pH 10.7) and 0.05MCaAc$_2$. at 25°.
—○— amylase activity, --□-- number of ε-DNP-lysine.

The possibility that denatured TAA might be dinitrophenylated with DNBS faster than the native enzyme was checked by comparing the dinitrophenylation rate of urea-denatured TAA with that of the native protein. No difference was, however, detected between them.

Summarizing all the findings described in this paper, it may be concluded that the ε-amino groups of lysine residues do not play any essential role for the emergence of the activity of TAA, but some special phenol groups of tyrosine residues are more closely connected to the activity.

SUMMARY

1. Fluorodinitrobenzene was reacted with Taka-amylase A. The amylase activity seems to be completely lost when 2 moles of tyrosine and 5 moles of

lysine residues are dinitrophenylated by the reagent.

2. When dinitrobenzene sulfonate was reacted with Taka-amylase A at pH 10.7, 11 out of the total 22 lysine residues in the amylase molecule were dinitrophenylated. About 60 per cent of the original activity was still remained under these conditions.

3. It was suggested that ε-amino groups of lysine residues may not be essential for the amylase activity but some special phenolic groups of tyrosine may be more closely connected to the activity.

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SYNTHESIS OF L-CYSTEIC ACID AMIDE AND ITS HYDROLYSIS BY LEUCINE AMINOPEPTIDASE

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A synthetic substrate containing cysteic acid has not been tested by any proteolytic enzymes so far. However, because interest is increasing in the use of leucine aminopeptidase for the determination of N-terminal sequence of proteins and peptides (*1*, *2*), it is desirable to examine the susceptibility of cysteic acid amide to this enzyme.

L-Cysteic acid amide has already been synthesized in du Vigneaud's laboratory by a method which utilizes the following reactions (*3*): dicarbobenzoxycystine→dicarbobenzoxycystinyl chloride→dicarbobenzoxycystinamide→cysteinamide→cystinamide→cysteic acid amide. It should be noted that their procedure was considerably intricate and the yield was very poor and the over-all yield was only 17 per cent based on dicarbobenzoxycystine.

It was found that it's better yield was obtained by the present authors with less manipulation through esterification of L-cysteic acid to produce cysteic acid ethyl ester and its amidation to cysteic acid amide; the over-all yield has been made up to 76 per cent based on cysteic acid. The cysteic acid amide thus obtained was used throughout the enzymatic experiment.

The present communication also includes the results of hydrolysis rate of the amide by leucine aminopeptidase.

EXPERIMENTALS

Synthesis of the Compounds

L-*Cysteic Acid Ethyl Ester***—L-Cysteic acid monohydrate (10.0 g.) (*4*) was suspended in 800 ml. of absolute ethanol, and dry HCl was passed into it at room temperature to saturation. The solution was refluxed for 30 minutes and concentrated *in vacuo* to dryness. The residue was suspended again in 500 ml. of ethanol, and the same procedure was repeated. The solvent was removed *in vacuo* repeatedly with ethanol, and the residual crystals were collected with an aid of small amount of ethanol, washed with ether, and finally dried in air. Recrystallization was done from hot methanol, and the crystalline monohydrate was obtained as needles. Yield, 10.7 g. (93 per cent); $[\alpha]_D^{20}$+4.1° (c 2, in water).

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** In the same manner, cysteic acid methyl ester was obtained in crystalline state, but hygroscopic.

The compound lost 8.4 per cent of its weight upon drying for 2 hour *sin vacuo* at 110° (calculated for $C_5H_{13}O_6NS \cdot H_2O$, H_2O 8.4 per cent); m.p. 238–240°.

$C_5H_{13}O_6NS$ (197.2) Calcd. C 30.4, H 5.6, N 7.1
Found C 30.3, H 5.8, N 7.1

L-*Cysteic Acid Amide*—L-Cysteic acid ethyl ester monohydrate (4.3 g.) was dissolved in 60 ml. of methanol which had been saturated at 0° with dry NH_3. After standing it for 2 days in a pressure bottle at room temperature, the solution was concentrated *in vacuo* to dryness, and the residual crystals were collected with an aid of ethanol. The product was recrystallized as prisms from hot water. The air-dried crystals had no crystal water. Its melting point was not definite; the sample decomposed gradually over about 230°. Yield, 2.7 g. (81 per cent); $[\alpha]_D^{20}$+6° (c 0.32, in water).

$C_3H_8O_4N_2S$ (168.2) Calcd. C 21.4, H 4.8, N 16.7
Found C 21.6, H 4.7, N 16.5

Mueller *et al.* reported melting point for this compound as 272° (decomp.), but made no mention of specific rotation (*3*).

Enzyme and Methods

Preparation of Leucine Aminopeptidase—A partially purified leucine aminopeptidase solution (Step 4) was prepared as described (*5*). The purity of the enzyme was tested by its activity towards L-leucinamide. At 38° and pH 8.0 (0.1 *M* Tris buffer), the preincubated enzyme hydrolyzed 0.05 *M* L-leucinamide with a proteolytic coefficient (C_1) of 2.6. The activity (C_1) of Step 4 preparation for 0.05 *M* L-leucinamide was reported to be 2.5∼3.5 at 40° and pH 8.0 (*5*).

Methods—The assay was performed as follows. The enzyme preparation was preincubated in 0.1 *M* Tris buffer of pH 8.0 with 0.008 *M* $MnCl_2$ for 1 hour at 38°. An aliquot of the activated enzyme solution was added to a 2 or 5 ml. assay flask containing $MnCl_2$ and a substrate in specified concentrations buffered with 0.02 *N* NaOH and Tris at pH 8.0. Solution was made up to 2 or 5 ml. by water, and the final concentrations of Tris and $MnCl_2$ were 0.1 *M* and 0.008 *M*, respectively. It was incubated at 38.0°, and the hydrolysis rates were followed by means of the Conway microdiffusion method (*6*).

First order proteolytic coefficients (C_1) were estimated from $C_1 = K_1/E$, where K_1 is the first order velocity constant calculated in decimal logarithmus and E is the protein concentration in mg. of protein N per ml. of test solution. Zero order proteolytic coefficient (C_0) were determined from $C_0 = K_0/E$, where K_0 is the zero order velocity constant calculated as per cent hydrolysis per minute.

RESULTS AND DISCUSSION

In order to compare relative rate of hydrolysis of L-cysteic acid amide to that of L-leucinamide, both substrates at 0.01 *M* and 0.025 *M* concentrations were tested with the partially purified leucine aminopeptidase. Cysteic acid amide at 0.05 *M* was tested, because it was sparingly soluble at this concentration in buffer at pH 8.0. As seen from Table I, the hydrolysis of leucinamide followed first order kinetics, while that of cysteic acid amide has zero order kinetics. The average values of C_0 of cysteic acid amide were shown in Table II. For the comparison of relative rates of hydrolysis, zero order proteolytic coefficients (C_0), which are shown in Table II, were calculated from the rates of 30 per cent hydrolysis for leucinamide as Smith and Spackman have already suggested in their paper (*7*). It should be noted that cysteic acid amide was hydrolyzed at a much slower rate than is leucinamide.

TABLE I

Hydrolysis of Amino Acid Amides by Leucine Aminopeptidase

pH 8.0 (0.1 *M* Tris buffer); 0.008 *M* $MnCl_2$; temperature, 38°.

Substrate	Substrate concentration (*M*)	Enzyme concentration (10^{-3} *mg. protein N per ml.*)	Time (*min.*)	Hydrolysis (*per cent*)	C_0	C_1
L-Leucinamide	0.025	0.67	16	10.6		4.5
			32	18.8		4.2
			53	28.0		4.0
			72	37.1		4.2
			94	44.9		4.1
	0.01	0.67	18	16.4		6.4
			34	27.8		6.2
			54	40.8		6.3
			74	50.7		6.2
			97	61.0		6.3
L-Cysteic acid amide	0.025	120	64	7.7	0.87	
			122	14.6	0.85	
			180	21.6	0.88	
			244	25.1	0.86	
			305	32.6	0.89	
	0.01	143	70	9.4	0.94	
			126	17.7	0.98	
			190	24.4	0.90	
			255	43.1	1.18	
			310	46.8	1.06	

TABLE II

Relative Rates of Hydrolysis of Amino Acid Amides by Leucine Aminopeptidase

The value for L-leucinamide is given as 100.

Substrate	0.025 *M* concentration		0.01 *M* concentration	
	C_0	Relative rate	C_0	Relative rate
L-Leucinamide	800	100	1,210	100
L-Cysteic acid amide	0.87	0.11	1.01	0.083

Smith and Spackman have determined the proteolytic coefficients of a number of amino acid amides and peptides at pH 8.0 and 0.05 *M* substrate concentration using the highly purified leucine aminopeptidase (*7*). They found that glycinamide and tertiary leucinamide (*8*) were hydrolyzed especially slowly among the substrates tested. For example, glycinamide is hydrolyzed at 0.13 per cent of the rate of leucinamide. Table II indicates that cysteic acid amide is regarded as a poor substrate as glycinamide and tertiary leucinamide.

The results in Table II will also prove that cysteic acid should be removed very slowly by leucine aminopeptidase when the enzyme is applied for the determination of N-terminal sequences of oxidized proteins and peptides which contain cysteic acid as residues at N-terminal region.

SUMMARY

The improved method of synthesis of L-cysteic acid amide has been established, which utilizes the following reactions: (1) conversion of cysteic acid to cysteic acid ethyl ester; (2) amidation of the ester to yield cysteic acid amide.

Partially purified leucine aminopeptidase hydrolyzes cysteic acid amide at 0.11 per cent and 0.083 per cent at 0.025 M and 0.01 M substrate concentrations, respectively, of the rate of L-leucinamide.

The authors wish to thank Prof. S. Shibuya for his interest in this study.

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STUDIES ON THE CHEMICAL STRUCTURE OF BACTERIAL GLUTAMYL POLYPEPTIDES BY HYDRAZINOLYSIS

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During the investigation of the studies on the natural resistance against anthrax disease, the author found that dog liver extract hydrolyzed M-GPP* and S-GPP but not A-GPP (*1*). This fact indicates that A-GPP is not identical with the GPP of other two species in its chemical structure. In the previous paper it was shown that A-GPP was solely composed of D-Glu, but S-GPP and M-GPP of both D- and L-isomers (*2*). This is in accordance with evidences of the presence of L-isomers in S-GPP shown by Camp Detrick group (*3*, *4*) and by Bovarnick**.

For the clarification of the chemical structure, it is also necessary to elucidate the kind of linkage. Such investigations have been made by several workers (*5–13*). Above all, the presence of α-linkage of glutamic residue in A-GPP was persisted by Hanby and Rydon (*6*), but all recent investigations showed that γ-linkage was predominant in S- and A-GPP. The author performed hydrazinolysis of polypeptides derived from three species for the elucidation of their chemical structure. Hydrazinolysis is a method originated by Akabori *et al.* (*14*) for the estimation of carboxyl terminal of protein. When protein is treated with anhydrous hydrazine at 100°, carboxyl terminal amino acid (or acids) is liberated as a free amino acid, but the other amino acid residues as amino acid hydrazides. If GPP is subjected to hydrazinolysis, it is expected that α-linked Glu residue is converted to α-H, γ-linked residue to γ-H and $\alpha\gamma$-dilinked residue to $\alpha\gamma$-H respectively. This communication describes the observation on hydrozinolysis of GPP.

MATERIALS AND METHODS

Compounds Used—A-GPP (mol. wt. 56,200, by DNP-method) was prepared according to Hanby and Rydon (*6*) from the culture filtrate of *B. anthracis* (highly virulent strain "Vollum"). M-GPP (mol. wt. 87,000, by DNP-method) was obtained according to Bovarnick (*5*) from the hot water extract of encapsulated *B. megaterium*. Cultural conditions were described in the previous paper (*1*). Both organisms were kindly furnished by Prof. J. Tomcsik. S-GPP (mol. wt. 18,550) was presented through the kindness of Dr. M. Bovarnick. L-Glutamic acid α-hydrazide, L-glutamic acid γ-hydrazide, diDPN-glutamic acid α-hydrazide, diDNP-glutamic acid γ-hydrazide, triDNP-glutamic acid α,γ-dihydrazide were kindly provided by Prof. S. Akabori (Dept. Chem., Osaka Univ.), α-L-glutamyl L-glutamic acid by Dr. Shiba (Dept. Chem., Osaka Univ.), synthetic α-L-glutamyl polypeptide by Dr. Yuki (Dept. Chem., Osaka Univ.), and DL-isoglutamine by Dr. Sakamoto (Dept. Biochem., Osaka Univ.) respectively. L-Glutamic acid $\alpha\gamma$-dihydrazide was prepared as follows: diethyl glutamate (*15*) was dissolved in absolute ethanol and two moles of hydrazine hydrate were added, the mixture was allowed to stand at room temperature over night and then put into the ice box, and then crystals separated were recrystallized from water-ethanol. m.p. 145–147°. *Analysis*: $C_5H_{13}O_2N_5$, Calcd.; C 34.28, H 7.48, N 39.98, Found; C 33.91, H 7.12, N 41.01. L-Pyrrolidone carboxylic acid hydrazide was prepared according to Angier *et al.* (*16*). m.p. 114–115° (Angier, 114–115°). *Analysis*: $C_5H_9O_2N_3$, Calcd.; C 41.95, H 6.34, N 29.36. Found; C 42.49, H 6.37, N 29.52. DL-Pyrrolidone carboxylic acid was obtained by the Abderhalden's method (*17*) m.p. 180–182° (Abderhalden, 181–182°). Anhydrous hydrazine was obtained by distillation of hydrazine hydrate with CaO using a copper flask (*18*).

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* The abbreviations used are as follows: GPP, glutamyl polypeptide; A-GPP, GPP of *B. anthracis*; M-GPP, GPP of *B. megaterium*; S-GPP, GPP of *B. subtilis*; Glu, glutamic acid; α-H, glutamic acid α-hydrazide; γ-H, glutamic acid γ-hydrazide; $\alpha\gamma$-H, glutamic acid $\alpha\gamma$-dihydrazide; p-H, pyrrolidone carboxylic acid hydrazide; PCA, pyrrolidone carboxylic acid; DNP, dinitrophenyl; Glu-Glu, glutamyl glutamic acid.

** Bovarnick, M., Personal communication.

Estimation—α-H, γ-H, $\alpha\gamma$-H and Glu were estimated by the Troll and Cannan's method (*19*) and p-H by the Somogyi's method (*20*), after paper electrophoresis. The area of paper retaining a substance to be tested was cut, placed in a test tube and estimated. PCA was determined as Glu after hydrolyzing by 6 *N* HCl at 100° for 6 hours.

General Procedure of Hydrazinolysis—Two to ten mg. of GPP were dissolved in 0.5 ml. of hydrazine in a test tube sealed or equipped with a $CaCl_2$ tube and heated for appropriate periods. After cooling, the excess of hydrazine was evaporated *in vacuo* on H_2SO_4. Residual material was subjected to appropriate analytical methods.

EXPERIMENTALS AND RESULTS

Hydrazinolysis of GPP at 100° for 6 Hours

Dinitrophenylation of Hydrazinolysate and Subsequent Partition Column Chromatography—Hydrazinolysate was dinitrophenylated by the oridinary method and separated to acidic and neutral fractions by ethyl acetate extraction according to Ohno (*21*). Acidic fraction containing DNP-derivatives of α-H and γ-H was subjected to partition chromatography. DNP-derivatives of α-H and γ-H which were separated on column were hydrolyzed with 6 *N* HCl at 100° for 6 hours respectively and the resulting DNP-Glu was estimated by a Beckman spectrophotometer (Model DU) at 360 mμ after separation on column. Neutral fraction containing DNP-derivatives of $\alpha\gamma$-H was hydrolyzed under the same condition without chromatograyhic separation and then the value of $\alpha\gamma$-H was given by estimating the DNP-Glu released. Chromatograms and their conditions were shown in Fig. 1 and the results were given in Table I. As the values shown in Table I were not compensated for loss during the treatment, the amounts of hydrazides were small. However, the amount of αγ-H was unexpectedly large in comparison with α- and γ-H.

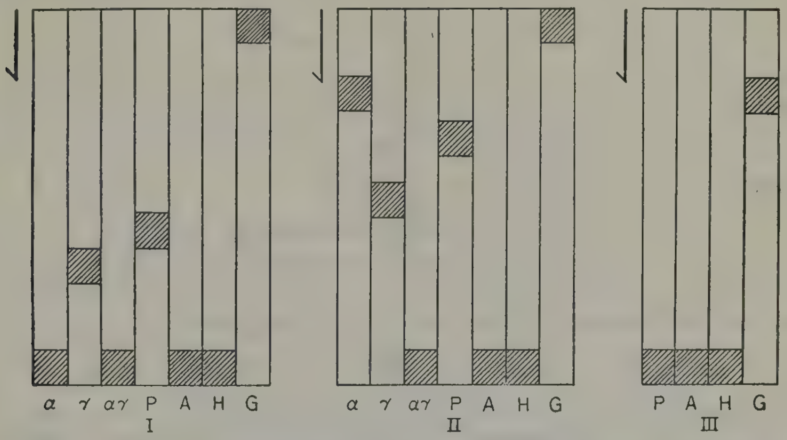

FIG. 1. Partition chromatographic picture of DNP-derivatives of glutamic acid and its hydrazides. Stationary phase is celite 545 buffered at pH 6.9 (I), pH 9.0 (II) and pH 5.3 (III) with 0.2 *M* phosphate buffers. Moving phase is ethyl acetate saturated with buffer. *α*, *γ*, *αγ* and G are DNP-derivatives of *α*-, *γ*-, *αγ*-H and Glu. P, A and H are dintrophenol, dinitroanilin and dinitrophenyl hydrazine respectively.

TABLE I

The Amounts of Hydrazides Found in Hydrazinolysate of GPP (by DNP-Method)

Materials	α-H (*per cent*)	γ-H (*per cent*)	αγ-H (*per cent*)
A-GPP	0.065	5.74	12.2
M-GPP	0.035	3.89	22.1
S-GPP	0.182	4.86	13.0

Per cent represents the value to total glutamic acid residue.

Paper Chromatography of Hydrazinolysate—On the paper chromatograms γ-H and αγ-H appeared and furthermore p-H was found, but α-H disappeared (Fig. 2).

Paper Electrophoresis of Hydrazinolysate—The conditions proposed by Narita were used (*22*). In the first electrophoretic run, α-, γ- and p-H were separated from αγ-H and Glu. The mixture of three hydrazides was then separated to each hydrazide respectively in the second run. Paper electrophoretic conditions and pictures were given in Fig. 3. As shown in Fig. 3, γ-H, αγ-H and p-H were found in GPP hydrazinolysate but α-H was not.

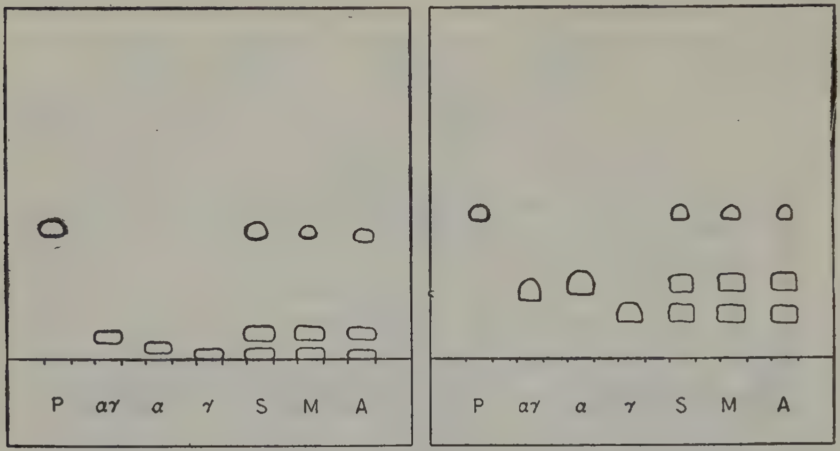

FIG. 2. Paper chromatogram of GPP hydrazinolysate (100°, 6 hours). Solvent: right, *n*-butanol 40, acetic acid 10, water 10, left: phenol 75, pyridine 10, lutidine 40, water 25, ethanol 60. Color reagent is ammoniacal silver solution, α, γ, αγ and p are α-, γ-, αγ- and p-H. A, M and S are hydrazinolysate of A-, M- and S-GPP.

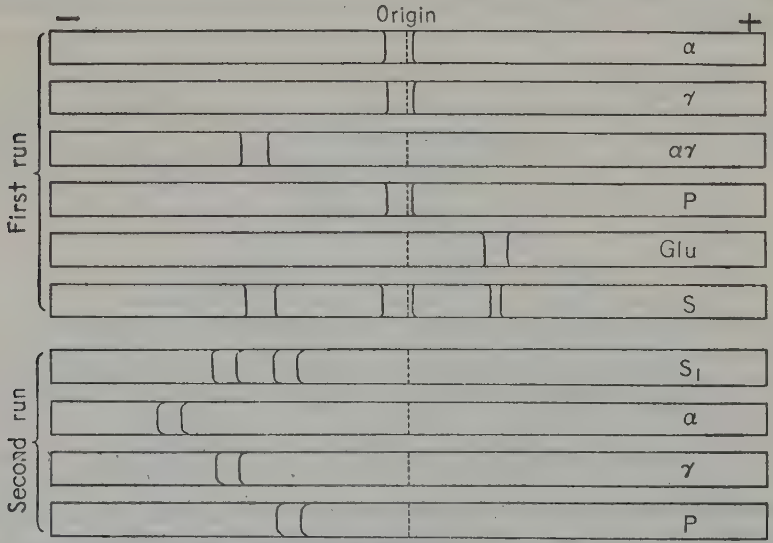

FIG. 3. Paper electrophoretic diagrams. The 1st run: pH 6.48 buffer (lutidine 36.5 ml., 1 *N* acetic acid 185 ml., H_2O 3.86 liter). The 2nd run: pH 2.68 buffer (0.5 *N* Acetic acid). Each run was carried out for 2 hours (500 volts) using Toyo filter paper No. 51A (2×40 cm.). α, γ, αγ and p: α-, γ-, αγ- and p-H. S: GPP hydrazinolysate (100°, 6 hours). S_1: 2nd run of center fraction (non-mobile) in S.

From the fact that a considerable amount of αγ-H and p-H was found in GPP hydrazinolysate, it was supposed that there might be branchings, amides or rings, especially pyrrolidone rings, in the GPP structure. However, such structure was not truely believed from the following experiments.

Various Analysis of S-GPP

Determination of Amide N—After treatment of S-GPP by HCl, determination of NH_3 was carried out by the Conway's microdiffusion method (*23*). As the following Table II shows, amide N was not found.

TABLE II

Determination of Amide N of S-GPP

Hydrolysis	Evolution of NH_3
2 *N* HCl, 100°, 3 hours	0
2 *N* HCl, 100°, 16 hours	0

Titration with Sodium Hydroxide—S-GPP was suspended in water and titrated with NaOH solution using phenolphthalein as indicator. When slight pink color developed, S-GPP had been completely dissolved in the solution. Since 12.9 mg. of S-GPP was shown to be equivalent to 5.30 ml. of 0.0187 *N* NaOH, the equivalent of S-GPP was calculated to be 130 and this value is in good agreement with glutamic acid residue ($C_5H_7O_3N$, 129), This fact shows that the numbers of free carboxyl group in S-GPP is almost equal to those of glutamic acid residue, and therefore there might be no ring structure in GPP.

TABLE III

Ratio of N-Terminal to C-Terminal of S-GPP

GPP (*mg.*)	N-terminal Glu (μM)	C-terminal Glu (μM)	C-terminal/N-terminal
12.9	0.708[1]	—	—
12.9	—	1.19[2]	1.68
12.9	—	1.00[3]	1.41

1) This value gives the molecular weight of 18,200.
2) By the paper electrophoretic method.
3) By the DNP-method.

Ratio of Amino (N-) Terminal to Carboxyl (C-) Terminal of S-GPP—As amino terminal is one in one GPP molecule, if there are some branchings, the value of C-terminal per N-terminal should be more than one. N-terminal was determined by the DNP-method, *i.e.* GPP was dinitrophenylated and hydrolyzed by 6 *N* HCl for 6 hours at 100°. The resulting DNP-Glu was estimated after celite 545 chromatographic separation. C-terminal was determined by hydrazinolysis, that is, GPP was treated with anhydrous hydrazine at 100° for 6 hours. After evaporation of hydrazine, Glu in hydrazinolysate was determined by DNP-method and paper electrophoretic method. The results shown in Table III suggest that there seems to be no branching.

Elementary Analysis—Percentages of C, H and N were in good agreement with $(C_5H_7O_3N)_n$ Calcd.; C 46.51, H 5.46, N 10.85, Found; C 46.70, H 5.32, N 10.55.

From these four experimental results it is assumed that there are not such structures in GPP originally as one which gives $\alpha\gamma$-H or p-H by hydrazinolyzing. But it is rather possible for $\alpha\gamma$-H and p-H to be produced from the other reason, *e.g.* from the subsequent decomposition by hydrazine, after intramolecular ring closures of GPP which might take place because of dehydration by anhydrous hydrazine. Indeed such a ring closure was found by V. Bruckner *et al.*, when synthetic α-glutamyl polypeptide was treated with acetic anhydride (*24*).

Hydrazinolysis of S-GPP under Various Conditions

Now hydrazinolysis was carried out at lower temperature than 100° or by using hydrazine hydrate. The all experiments to determine fission products described in this section and below were performed by the paper electrophoresis. Although the results described in this section were obtained using S-GPP, in the case of A- and M-GPP the results were also almost similar. As the temperature of hydrazinolysis being reduced from 100° to 72°, 50°, 43°, 37° and 25°, formations of $\alpha\gamma$-H and p-H were decreased. And after

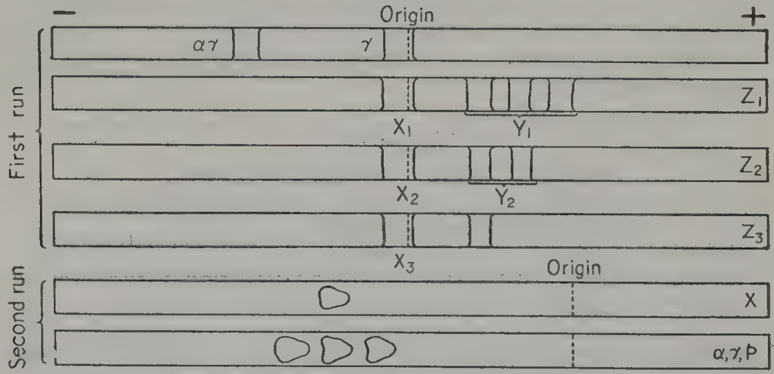

FIG. 4. Paper electrophoretic diagrams of S-GPP hydrazinolysate. Electrophoretic conditions are the same with Fig. 3. Z_1: GPP-hydrazinolysate (37°, 24 hours), Z_2: hydrazinolysate of Y_1 (37°, 24 hours), Z_3: hydrazinolysate of Y_2 (37°, 24 hours), X: eluate of X_1, X_2 or X_3. Color development: by ammoniacal silver solution.

being treated for 24 hours at 37°, the formation of αγ- and p-H were not found but only γ-H appeared. However, under such lower temperature hydrazinolysis was not completed and hydrazides of shorter glutamyl peptides were found. When these were eluted, dried and retreated by anhydrous hydrazine at 37° for 24 hours, γ-H and incompletely hydrazinolyzed products

TABLE IV

Hydrazinolysis of S-GPP under Various Conditions

Temperature (°*C*)	Hydrazine	Time (*hour*)	Product (percentage to total glutamic acid residues)				
			α-H	γ-H	αγ-H	p-H	Pep-H*
100	Anhydrous	6	–	+++ (23.5)	+++ (26.1)	+++ (26.0)	–
”	Hydrate	6	–	++ (15.7)	+ (5.2)	+ (5.4)	
72	Anhydrous	6	–	++	+	+	+
”	”	14	–	+++	++	++	
”	Hydrate	19	–	++	+	+	++
”	50% hydrate	19	–	+	tr	tr	+++
50	Anhydrous	47	–	++	tr	tr	+++
”	”	93	–	+++	+	+	++
43	”	240	–	+++	+	+	++
37	”	24	–	++ (7.4)	–	–	+++
”	”	40	–	++ (16.3)	–	–	++
”	”	260	–	++++ (38.7)	+	+	++
”	”	24	–	+ (5.3)	–	–	+++[1]
”	”	24[1]	–	++ (12.1)	–	–	++[2]
”	”	24[2]	–	+	–	–	+
”	”	240	–	++++ (33.0)	+ (2.9)	+	++[3]
”	”	240[3]	–	+ (5.7)	+ (0.7)	+	+[4]
”	”	240[4]	–	+ (2.2)	+ (0.2)	tr	+[5]
”	”	240[5]	–	+ (2.2)	tr	tr	–
25	”	30 days	–	++ (10.2)	+ (0.3)		+++

* Incompletely decomposed peptide hydrazide. 1, 2, 3, 4 and 5 in the column of time show hydrazinolysis of incompletely decomposed peptide hydrazides numbered in the column of Pep-H respectively. ++++ : maximum color development on paper, – : not detectable, tr : trace.

were seen again (see Fig. 4). Although the use of hydrazine hydrate or the treatment at 25° decreased the formations of $\alpha\gamma$- and p-H, these treatments were not preferable as considerable incomplete decomposition occurred. And α-H was not found in all experiments. The results are summarized in Table IV. These results show that the formation of $\alpha\gamma$- and p-H will be kept off, if a suitable condition is taken. Considerable occurrences of $\alpha\gamma$- and p-H in the early experiment seem to be due to the secondary decomposition of modified GPP resulted from dehydration by anhydrous hydrazine.

Occurrence of PCA during Hydrazinolysis of GPP

As the value of γ-H did not nearly approach 100 per cent, even when prolonged and repeated treatments were performed, the mutual transformation between γ-H and PCA was considered. When γ-H or PCA was treated with anhydrous hydrazine, PCA or γ-H occurred respectively. And in fact, PCA was found in hydrazinolysate of GPP as shown in Figs. 5 and 6.

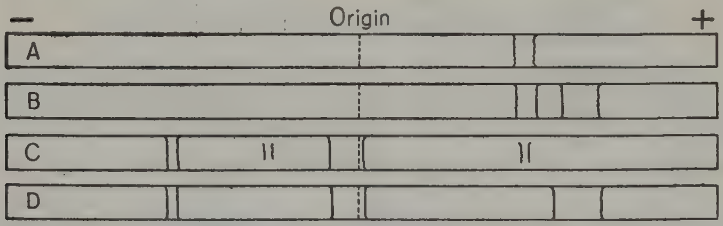

FIG. 5. Detection of PCA in GPP hydrazinolysate (by paper electrophoresis). A: Glu and PCA, sprayed by ninhydrin butanol solution. B: Glu and PCA, by Cl_2-KI-starch method (*25*). C: hydrazinolysate of GPP (37°, 50 days), by ninhydrin. D: hydrazinolysate of GPP (37°, 50 day), by Cl_2-KI-starch method. pH 6.48 buffer was used.

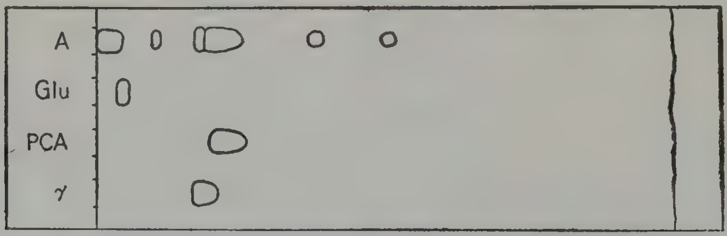

FIG. 6. Detection of PCA in GPP hydrazinolysate (by paper chromatography). A: GPP hydrazinolysate (37°, 18 days). Solvent: isopropanol 80, 0.03 *M* pH 6.7 phosphate buffer 20. Color development: Cl_2-KI-starch method.

Prolonged Hydrazinolysis of GPP at 37°

For complete hydrazinolysis, GPP were treated by anhydrous hydrazine

Table V

Prolonged Hydrazinolysis of GPP at 37°

Materials	Time (*day*)	Product (percentage to total glutamic acid residues)						
		α-H	γ-H	αγ-H	p-H	Pep-H	PCA	Unknown substance
A-GPP	18	—	+++ (21.0)	+ (1.8)	+	+	+++	
	50	—	++++ (52.0)	+ (4.6)	+	—	+++ (28.8)	+
M-GPP	18	—	++++ (37.0)	+ (0·7)	+	+	+++	
	50	—	++++ (53.3)	+ (8.6)	+	—	+++ (29.1)	+
S-GPP	18	—	++++ (36.8)	+ (3.8)	+	+	+++	
	50	—	++++ (55.4)	+ (14.7)	+	—	+++ (29.6)	+
γ-H	50	—	++++ (43.5)	+ (8.4)	+	—	+++ (26.3)	+

Table VI

Hydrazinolysis of Glutamine, Isoglutamine, α-Glu-Glu and α-GPP

Compounds	Temperature (°C)	Time (*hour*)	Products						
			α-H	γ-H	αγ-H	p-H	Pep-H	Unknown substances	PCA
Glutamine	37	24	—	++++	—	—			+
	”	48	—	+++	tr	tr			++
Iso-glutamine	37	24	+++	—	—	—			—
	”	48	+++	—	tr	tr			—
α-Glu-Glu	37	68	++	—	tr	tr		+	
	”	11 (*days*)	++	—	tr	tr		+	
α-GPP	100	6	++	—	++	++		+	
	37	24	+	++*	tr	tr	+++[1]	+	
	”	24[1]	+	++*	tr	tr	++	+	
	”	7 (*days*)	+	++*	tr	tr		+	
	”	50 (*days*)	+	++*	+	+		+	—

* This was shown to be the same as γ-hydrazide in electrophoretic behavior, but does not seem to be true γ-H because PCA was not found in hydrazinolysate treated for 50 days. 1 in the column of time shows hydrazinolysis of peptide hydrazide 1.

for 18 and 50 days at 37°. After 18 days, incompletely decomposed peptide hydrazide was still found. But 50 days after, hydrazinolysis was shown to be complete and so γ-H and PCA were estimated. For the purpose of comparison, γ-H was treated in the same way. The results given in Table V show that γ-H plus PCA is estimated to be 80.8–85.0 per cent after 50 days hydrazinolysis of GPP, and from γ-H itself, 79.8 per cent of γ-H plus PCA was recovered. And $\alpha\gamma$- and p-H were found not only from GPP but also from γ-H in approximately same amount.

Although repeated treatment for 24 hours was shown to hardly produce $\alpha\gamma$- and p-H, this was not used because some extent of hydrolysis of peptide hydrazides to peptides during elution was suspected.

Hydrazinolysis of Glutamine, Isoglutamine, α-Glutamyl Glutamic Acid and α-Glutamyl Polypeptide

Now it is necessary to hydrazinolyze synthetic γ-glutamyl polypeptide. But the author could not obtain it. Therefore, glutamine was used instead of it and isoglutamine, α-Glu-Glu and α-GPP were also hydrazinolyzed for comparison. As the results shown in Table VI, γ-H was found only from γ-linked glutamyl compound, whereas α-H only from α-linked compounds. And besides, on the hydrazinolysis of α-linked compounds unknown substances occurred and the yield of α-H seemed to be low.

From the experimental results described above, it may be concluded that glutamyl polypeptides produced by *B. anthracis*, *B. megaterium* and *B. subtilis* are almost all composed of γ-linkage, whatever α-H was detected by the DNP-method in early experiments. Because it is supposed that occurrence of this hydrazide might be due to the partial hydrolysis of $\alpha\gamma$-H during dinitrophenylation.

DISCUSSION

It was later found that γ-H itself gave $\alpha\gamma$-H when treated with hydrazine at 100°. Therefore, it is still obscure whether the formation of $\alpha\gamma$-H is due to the further hydrazinolysis after intramolecular ring closure of GPP or to subsequent formation from γ-H once resulted from direct hydrazinolysis. The formation of p-H can be explained by the muturl transformation between $\alpha\gamma$-H and p-H, which was also proven.

Although the author has been carrying out these experiments, assuming that A-GPP may be different from M- and S-GPP in the kind of linkage, the results given by hydrazinolysis did not show a particular difference between these peptides. Therefore, a difference of chemical structure between these peptides seems to be only whether they contain L-Glu or not. Accordingly, the fact that the dog liver extract can hydrolyze M- and S-GPP is probably due to the fact that these peptides are composed not only by D-isomer but also by L-isomer. As evidently the enzyme contained in the extract can not hydrolyze γ-D-Glu-D-Glu linkage, it is now interesting to study whether the enzyme is able to split each γ-linkage of D-Glu-L-Glu, L-Glu-D-Glu and L-Glu-L-Glu or any one only. The enzyme which hydrolyzes S-GPP was found by Thorne *et al.* (*4*) in the culture filtrate of *B. subtilis* and by Kream *et al.* (*26*) in human hemolysate and tissue extracts. The enzyme found in dog liver extract is probably identical with that of Kream *et al.*, but not with that of Thorne *et al.*, because the latter can hydrolyze A-GPP (*27*).

The fact that pathogenic *B. anthracis* produces a glutamyl polypeptide which has aggressive properties (*9*) and resists the hydrolytic action of the enzyme, whereas non-pathogenic *B. megaterium* and *B. subtilis* produce another glutamyl polypeptide which is easily hydrolyzed, seems to suggest some relationship between pathogenicity of bacteria and behaviours of peptides against enzymic action.

SUMMARY

Glutamyl polypeptides by *B. anthracis*, *B. megaterium* and *B. subtilis* were subjected to hydrazinolysis in order to elucidate the form of linkage of these polypeptides. Hydrazinolysis of these peptides at 100° gave not only glutamic acid γ-hydrazide but also considerable amounts of glutamic acid $\alpha\gamma$-dihydrazide and pyrrolidone carboxylic acid hydrazide which seemed to be secondary products. When prolonged hydrazinolysis was carried out at low temperature, the amount of γ-H increased, while those of $\alpha\gamma$- and p-H decreased. On hydrazinolysis at 37° for 50 days, major products were γ-H (52–55 per cent) and PCA (29 per cent) which was derived from γ-H. α-Hydrazide was not found in all experiments. These facts suggest that these glutamyl polypeptides are almost solely composed of γ-linkage.

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CATALYTIC HYDROGENOLYSIS OF DESAMINOCANAVANINE

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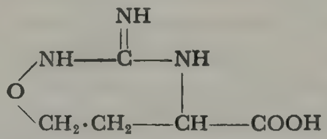
In 1937, Kitagawa and Tsukamoto reported the formation of a new compound from canavanine with a loss of ammonia (*1*). The evidence presented in that study indicated that the new compound should be designated as "desaminocanavanine", and the following constitutional formula was proposed provisionally to this compound.



With regard to the desaminocanavanine, however, no other paper has been published until now except a biological study by Appel (*2*).

In this paper a further evidence obtained from a catalytic hydrogenolysis of desaminocanavanine will be presented which equally justifies its structure suggested by Kitagawa and Tsukamoto. Some observations on the properties of the hydrogenolysis product will also be reported.

MATERIALS AND METHODS

Desaminocanavanine was isolated from jack bean meal together with canavanine. It was also able to prepare from canavanine as described by Kitagawa *et al.* (*1*). Homoserine was a commercial product. Palladium charcoal was prepared from active charcoal and palladium chloride by the ordinary method.

For the detection of desaminocanavanine and its related compounds, a descending paper chromatography devised by the author (*3*) was carried out by using several color reactions such as the Sakaguchi, alkaline nitroprusside-ferricyanide, the Jaffe and ninhydrin tests.

EXPERIMENTS AND RESULTS

Hydrogenolysis of Desaminocanavanine and Anhydride Formation from the Reaction Product—It is known that canaline is catalytically hydrogenolyzed to homoserine and ammonia (*4*). Recently, it was found that certain strains of bacteria have an ability to degrade canavanine enzymatically, with the formation of homoserine and guanidine (*5*). Furthermore, it was reported that canavaninosuccinate and canavanine can also be catalytically hydrogenolyzed at the same O-N linkage as in the case of canaline, with the formation of guanidinosuccinate and guanidine, respectively, in addition to homoserine (*6*).

In view of the results obtained with canaline, canavanine and canavaninosuccinic acid, the catalytic hydrogenolysis of desaminocanavanine was tested, just the same cleavage being expected since an O-N linkage also exists in the postulated structure of the latter. It was found that this compound was easily hydrogenolyzed at room temperature in the presence of palladium charcoal, when either aqueous or acetic acid solution of the compound was shaken with hydrogen gas. The reaction was practically completed within 2 hours. No reaction occurred in the absence of hydrogen.

TABLE I

Stoichiometric Absorption of Hydrogen by Desaminocanavanine

Exp. No.	Solvent	Sample	Hydrogen uptake
1	Acetic acid	92.8 *mg.* (0.58 mM)	13.95 *ml.* (0.57 mM)
2	Water	111.5 *mg.* (0.70 mM)	16.90 *ml.* (0.69 mM)

The mixtures consisting of about 0.6–0.7 mM of desaminocanavanine, 100 mg. of palladium charcoal and 30 ml. of acetic acid or water were shaken with hydrogen gas for 3 hours at 23° and 761 mm. Hg.

As shown in Table I, one mole of hydrogen was taken up per mole of desaminocanavanine in each case. The paper chromatographic analysis of the reaction product indicated the presence of a new guanidino compound which is considered to be *N*-amidinohomoserine (α-guanidino-γ-hydroxybutyric acid). With a solvent mixture composed of *n*-butanol-acetic acid-pyridine-water (4 : 1 : 1 : 2), this compound showed an R_f value (0.28) which is smaller than that of desaminocanavanine (0.44). For further confirmation, the new guanidino compound was isolated and submitted to analysis as follows.

Through a mixture containing 1 g. of desaminocanavanine, 1 g. of palladium charcoal and 250 ml. of water hydrogen was passed for 3 hours and then the mixture was filtered. The filtrate plus washings were concentrated to a small volume under reduced pressure. When the concentrate was added dropwise with about five times its amount of ethanol, crystals appeared. They were recrystallized from 80 per cent aqueous ethanol and dried *in vacuo*. The yield was 780 mg. It decomposed at 245–260° with sintering and showed a rotation, $[\alpha]_D^{19} -16.5°$ (2 per cent in water). The analytical data agreed well with *N*-amidinohomoserine.

$C_5H_{11}O_3N_3$: Calcd. C 37.25, H 6.88, N 26.08
Found C 37.42, H 6.82, N 26.00

By heating in an acid solution, this compound was transformed into a compound which gave positive reactions with alkaline nitroprusside-ferricyanide and the Jaffe reagents, but not with the Sakaguchi reagent. This fact led to the view that the compound was converted by this treatment into its anhydride of the structure analogous to creatinine. The view was supported paperchromatographically and analytically.

100 mg. of amidinohomoserine were dissolved in 10 ml. of acetic acid and heated with refluxing for an hour. The resulting solution was evaporated under reduced pressure with occasional addition of water until crystals of the anhydride appeared. After washing with aqueous ethanol, the anhydride was recrystallized from the same hot solvent. The yield was 75 mg. It decomposed at 245-260° and was optically inactive. The analytical data agreed well with the composition as the anhydride of *N*-amidinohomoserine.

$C_5H_9O_2N_3$: Calcd. C 41.95, H 6.34, N 29.36
Found C 41.84, H 6.31, N 29.01

Hydrolysis of N-Amidinohomoserine—Heating of amidinohomoserine in an alkaline solution at 100° hydrolyzed it into ammonia and a compound identified as homoserine by paper chromatography. The R_f values between the commercial and the hydrolysis product in *n*-butanol-acetic acid-pyridine-water (4 : 1 : 1 : 2), ethanol-water (7 : 3) and phenol-water (4 : 1) agreed satisfactorily with each other and were 0.24, 0.48 and 0.49, respectively. The hydrolysis product was further confirmed by isolation as follows.

350 mg. of amidinohomoserine were refluxed for 6 hours in 10 ml. of water containing 1 g. of hydrated barium hydroxide. Ammonia was liberated during the heating and considerable amount of barium carbonate was formed. After cooling, the solution was saturated with carbon dioxide. The precipitate produced was filtered off and the filtrate was boiled with copper carbonate for 5 minutes. The resulting solution of homoserine copper salt which had been freed from insoluble matter was concentrated to a small volume on a boiling water-bath and allowed to stand overnight in the cold for obtaining the crystalline copper salt. After separation and washing three times with a small volume of water, the salt was dried *in vacuo*. The yield was 120 mg. It decomposed at 232–233°. To obtain free homoserine 115 mg. of the copper salt were treated with hydrogen sulfide in the usual manner. The yield was 70 mg. After recrystallization from water-ethanol mixture, it melted at 183°. No optical rotation was observed, presumably by the rasemization during the alkaline hydrolysis. The analytical data showed it to be homoserine.

$C_4H_9O_3N$: Calcd. C 40.33, H 7.62, N 11.76
Found C 40.17, H 7.77, N 11.92

Guanidination of Homoserine—As amidinohomoserine is a guanidino compound which can be derived from homoserine, the synthesis was undertaken from homoserine and *S*-methylisothiourea, and the synthetic product was compared with the hydrogenolysis product from desaminocanavanine.

An equimolar mixture of DL-homoserine, *S*-methylisothiourea sulfate and barium hydroxide was allowed to react for one week at room temparature.

The yield by this procedure was not good, however, the product was proved chromatographically to be identical with the hydrogenolysis product.

On the other hand, the anhydride of amidinohomoserine was synthesized by the cyanamide fusion method as follows.

A mixture of 200 mg. of DL-homoserine, 80 mg. of cyanamide and 2 ml. of water was heated at about 135° for an hour. After cooling, the solid was triturated with 5 ml. of water. The residue separated by filtration was washed with a small volume of cold water and recrystallized from hot water. The yield was 120 mg. The decomposition occured at 245–246°. The analytical results of the synthetic compound agreed well with the theoretical values of the anhydride of amidinohomoserine.

$C_5H_9O_2N_3$: Calcd. C 41.95, H 6.34, N 29.36
Found C 41.92, H 6.21, N 29.21

Reversible transformation of the anhydride to its hydrated form occurred gradually in a dilute alkaline solution at room temperature, as in the case of creatinine to creatine.

Paper chromatographic behaviors and color tests of desaminocanavanine and its related compounds are summarized in Table II.

TABLE II

R_f Values and Color Test of the Related Compounds to Desaminocanavanine

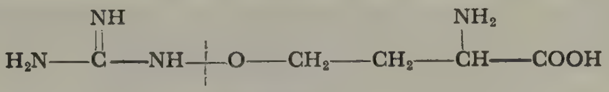
Related compound	R_f value	Color test		
	B. A. P. W.[1) (4 : 1 : 1 : 2)	Sakaguchi reaction	N-F[2) reaction	Jaffe reaction
Desaminocanavanine	0.44	+	+	−
Amidinohomoserine (isolated)	0.28	+	+	−
Amidinohomoserine anhydride (isolated)	0.54	−	+	+
Amidinohomoserine (synthetic)	0.28	+	+	−
Amidinohomoserine anhydride (synthetic)	0.54	−	+	+

1) *n*-Butanol-acetic acid-pyridine-water.
2) Alkaline nitroprusside-ferricyanide.

These results also show that the synthetic *N*-amidinohomoserine and its anhydride are identical with the isolated compounds respectively, supporting the proposed structure of desaminocanavanine.

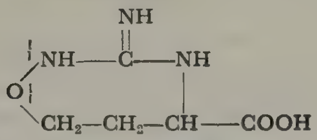
DISCUSSION

The experimental results obtained in this study afford additional supports to the previously assigned structure of desaminocanavanine. It became now evident that desaminocanavanine and canavanine can be catalytically hydrogenolyzed at the same O-N linkage, producing *N*-amidinohomoserine and homoserine plus guanidine, respectively, as indicated below.



$$H_2N-\overset{\overset{NH}{\|}}{C}-NH\vdots O-CH_2-CH_2-\overset{\overset{NH_2}{|}}{C}H-COOH$$

Canavanine



Desaminocanavanine

The result indicates that there is no difference between hydrogenolyses of such open chain and cyclic compounds.

Works now in progress show that desaminocanavanine is present in the jack bean and certain other higher plants together with canavanine. Up to the present its biological role, if any, is unknown.

SUMMARY

For confirming the proposed structure of desaminocanavanine, the compound was subjected to the catalytic hydrogenolysis with palladium charcoal and hydrogen gas in expectation of the cleavage at O-N bond. The hydrogenolysis product of desaminocanavanine, *N*-amidinohomoserine (α-guanidino-γ-hydroxybutyric acid), was isolated. Its characterization and identification were carried out by elementary analysis, anhydride formation, alkaline hydrolysis to homoserine and ammonia, and synthesis from homoserine.

From the fact that the hydrogenolysis product is only *N*-amidinohomoserine, the provisional cyclic formula of desaminocanavanine was found to be true.

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STUDIES ON THE FREE AMINO ACIDS AND RELATED COMPOUNDS IN THE BRAINS OF FISH, AMPHIBIA, REPTILE, AVES AND MAMMAL BY ION EXCHANGE CHROMATOGRAPHY

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(Received for publication, April 7, 1958)

The present study has been undertaken to detect whether there exists any difference in the free amino acids and their related compounds in the brains of the various classified animals.

The analysis of the free amino acids in the brains of several mammal species have so far been reported by many authors (*1–6*). These data were obtained chiefly by paperchromatographic procedure. The method is convenient, but is not exact in the quantitative analysis. Some of these data were obtained by the microbiological method (*2*, *7*, *8*). More satisfactory results were obtained, however, by using ion-exchange chromatographies (*9–11*).

In the present study, a significant difference in the distribution patterns of free amino acids and their related compounds in the brains of various animals of the vertebrate was found by means of ion-exchange chromatographic procedures.

EXPERIMENTALS

Materials—Catfishes, *Parasilurus asotus*, body weight about 300 g., twenty cerebra and midbrains were determined in December. Frogs, *Rana nigromaculata*, body weight 30–50 g.; forty total brains were determined in January in absolute hibernation. Tortoises, *Geoclemys reevesii*, body weight 400–800 g.; ten cerebra and midbrains were determined in January in relative hibernation. Hen, *Gallus domesticus*, body weight 2100 g.; a cerebrum and midbrain were determined in February. Male rats, *Rattus norvegicus var. albus*, body weight about 100 g.; five cerebra were determined in August.

Preparation of Brain Extracts—The animals were killed by decapitation, and brains were isolated, and immediately frozen in dry-ice acetone, weighed by torsion balance and the samples were ground in Potter-Elvehjem glass homogenizers with a 30-fold amount of 90 per cent ethanol. The ethanol precipitate was removed by centrifugation. The ethenol extract was evaporated to dryness over water bath at 50°. The dried residue was dissolved in 0.2 *N* sodium citrate buffer at pH 2.2 to be chromatographed on Dowex 50 column. The sample to be chromatographed on Dowex 2 column was dissolved in 0.2 *N* sodium acetate buffer at pH 5.5.

Chromatographic Analysis—The samples were analyzed on 150×0.9 cm. columns of Dowex 50-×4 by Moore and Stein's procedures (*10*), and on 15×0.9 cm. columns of Dowex

2-×8 for the determination of *N*-acetylaspartic acid by Tallan, Moore and Stein's procedures (*12*). Two ml. of each effluent fraction was collected. The concentrations of the amino acids in the effluent fractions were determined photometrically by the modified ninhydrin reagent (*13*) for 1 ml. of each fraction. Identification of the substances for the various peaks was aided by the use of paperchromatography (*14*). In the chromatography on Dowex 2 columns, remaining 1 ml. of each fraction was determined on *N*-acetylaspartic acid by modified ninhydrin method after acid hydrolysis. In the chromatographic procedures, taurine and phosphoethanolamine were overlapped. So the eluate containing these two substances was chromatographed on paper with 90 per cent methyl-cellosolve as the solvent system, and taurine and phosphoethanolamine were separated from each other quantitatively.

Total Amino Acid Nitrogen Contents of the samples were calculated by subtracting the ammonia values, measured by Conway's microdiffusion method (*15*) from values obtained by the modified ninhydrin method, read against a L-leucine standard.

RESULTS

An example of elution curve obtained from an extract of catfish brain by Dowex 50-×4 column is shown in Fig. 1.

Quantitative data on the twenty-five constituents and the total amino nitrogen contents in the brains of five animals are presented in Table I, together with the data reported by Tallan *et al.* on the brain of the cat (*11*).

Aspartic Acid, Glutamic Acid, Alanine and Serine—Generally, dicarboxylic acids, alanine and serine are found in characteristically lower concentrations in the brains of cold-blooded animals than in those of warm-blooded.

Other Amino Acids—Threonine, isoleucine, leucine and histidine are detected in the brains of catfishes in relatively larger amount. Glycine is found in the frog in relatively higher, and in the tortoise in much lower concentrations. The amount of threonine is relatively small in the frog and tortoise, which are both in hibernation. γ-Aminobutyric acid concentrations are in the same orders in the brains of five animals examined.

Related Compounds—Taurine is found in remarkably low concentrations in the brains of the frog and tortoise, which are both in hibernation, Urea is not detected in the brains of the catfish and hen. The concentrations of glutathione are relatively small in the brains of cold-blooded animals. Under the conditions of preparing the extracts, glutathione has not been completely oxidized, and therefore, it exists in the mixed form of the reduced and oxidized. This fact shows the varied concentrations of glutathione to be unreliable. *N*-Acetylaspartic acid has been determined in the brains of the catfish, the hen and the rat. It is detected only in a small amount in the catfish. The concentrations of glutamine found are not reliable. On the basis of the fact that the recovery of glutamine is about 25 per cent (*10*), corrected values are given in Table I, dividing the determined values by 0.25.

Unknown Substances—The substances designated as X_1, X_2,$\cdots$, X_6 (Table I) are not yet fully identified, and their relative concentrations are shown

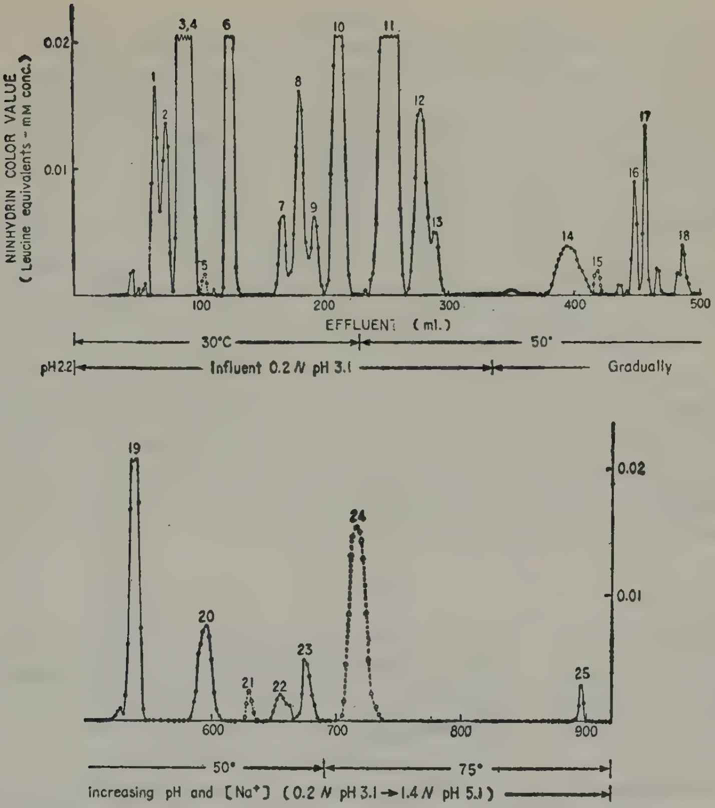

FIG. 1. The ninhydrin-positive components of an ethanol extract of catfish brain. The sample was analyzed on 150×0.9 cm. column of Dowex 50-×4. Taurine and phosphoethanolamine emerged from the column together and were estimated by paperchromatography. --○--; The substance detected in other animal brains and not found in catfish brain. The effluent was collected in 2 ml. fraction.

1. Glycerophosphoethanolamine, 2. X_1, 3. Phosphoethanolamine, 4. Taurine, 5. Urea, 6. X_2, 7. Aspartic acid, 8. Threonine, 9. Serine, 10. Glutamine, 11. Glutamic acid, 12. Glycine, 13. Alanine, 14. Glutathione, 15. X_3, 16. Isoleucine, 17. Leucine, 18. X_4, 19. γ-Aminobutyric acid, 20. Ammonia, 21. X_5, 22. Lysine, 23. Histidine, 24. X_6, 25. Arginine.

in leucine equivalent in mM. The relative concentrations of the unidentified substances vary in various animals. Especially, the very high concentrations of X_2 are found in the cold-blooded animals examined. The study of X_2 has been reported previously (*16*). The compound is acids hydrolizable and contains threonine, phosphoric ester and other ninhydrin positive substances. Besides these, a number of unidentified peaks are found in all the animal brains examined.

Total Amino Acid Nitrogen Content is found relatively in lower level in the brains of the frog and tortoise, which are both in hibernation. With the

TABLE I

The Free Amino Acids and Related Compounds in the Brains

(Values expressed in mg. per 100 g. wet weight)

Constituent	Catfish	Frog	Tortoise	Hen	Rat	Cat[1]
Amino N	50.5	38.1	30.3	50.5	43.5	—*
Glycerophosphoethanolamine	16.9	31.0	14.8	24.9	17.8	2.9
X_1**	0.041	0.012	—	—	—	—*
Phosphoethanolamine	17.6	21.0	10.2	45.8	19.8	41.9
Taurine	42.5	0.6	3.9	40.4	49.0	24.0
Urea	—	66	20	—	56	25
X_2**	0.719	0.316	0.428	0.057	0.011	—*
Aspartic acid	3.9	8.9	6.3	33.4	30.0	29.7
Threonine	7.9	1.8	0.5	4.2	3.6	2.6
Serine	2.9	2.1	1.3	12.9	10.1	7.6
Glutamine[3]	90	80	30	60	70	>50
Glutamic acid	74.2	64.2	68.0	158.2	147.0	128
Glycine	6.0	12.1	1.1	5.1	6.8	10.1
Alanine	2.2	2.1	1.1	4.3	5.0	8.4
Glutathione[3]	14.3	13.5	5.3	26.6	25.9	27.1
X_3**	—	—	—	0.013	—	—*
Isoleucine	2.8	1.3	—	1.4	1.6	1.2
Leucine	3.9	1.3	—	1.0	1.7	1.8
X_4**	—	—	0.019	—	—	—*
γ-Aminobutyric acid	18.8	28.2	17.9	28.0	22.1	23.4
X_5	—	—	0.023	—	—	—*
Lysine	3.0	1.2	—	1.7	1.8	2.0
Histidine	4.6	—	—	—	1.4	0.9
X_6**	—	0.190	0.146	0.052	—	—*
Arginine	1.3	1.2	1.8	—	1.9	1.4
N-Acetylaspartic acid	14	—*	—*	46	75	103[2]

—: Not detected. —*: Not examined. **: Unidentified substance.

Values expressed in mM concentration per 100 g. wet weight (in leucine equivalent).

1) By Tallan *et al.* (*11*). 2) By Tallan (*20*). 3) See text.

exception of these two species, it is found without any significant diversity among other animal species.

DISCUSSION

A small ammonia content and a low activity of glutamic dehydrogenase have been reported in the brain of the catfish by Takahashi (*17*). It may be of some interest that the amount of glutamic acid is relatively small in the brains of cold-blooded animals as evidenced in the present work. Aspartic acid concentrations are also very low in cold-blooded animal brains. Okumura (*18*) has demonstrated that the activity of glutamic decarboxylase in the brain of the catfish is very low, while it is very high in the brain of the frog. γ-Aminobutyric acid is found in the catfish in relatively low, and in the frog in relatively high concentrations. Remarkably small amount of taurine is found in the brains of the frog and tortoise in their hibernation. But, this may be a particular phenomena to be seen only during hibernation. According to our unpublished data, the taurine content found in the brain of the tortoise at the period not in hibernation is in the level of 20 mg. per 100 g. wet weight. Tallan (*19*) demonstrated that the brain of warm-blooded contains larger quantity of *N*-acetylaspartic acid than that of cold-blooded. The amount of the substance in the catfish brain is also small. Among the unidentified substances, X_2 is a most interesting one. Tallan *et al.* (*11*) reported the small peak of the unknown substance appearing on the effluent curves at 120 to 130 ml., which was encountered in all of the tissues of the cat. X_2 also appeared on the effluent curves at 120 to 130 ml.

SUMMARY

1. Comparative studies on free amino acids and their related compounds in the brains of various animals have been done by the methods of ion-exchange chromatographies.

2. The animals examined were the catfish, frog, tortoise, hen, and rat. The frog and tortoise were in hibernation.

3. The large differences were observed in the cold-blooded animals in comparison with the warm-blooded animals. Especially, lower values of glutamic acid, aspartic acid, alanine, serine and *N*-acetylaspartic acid were found in cold-blooded animals than in the warm-blooded. Unidentified substance X_2 containing threonine, phosphoric ester and other ninhydrin positive substances was found in a very large amount in the brains of the catfish, frog and tortoise.

4. The amount of γ-aminobutyric acid was much the same in all the vertebrates examined.

5. Extremely small amounts of taurine were obtained in the frog and tortoise, both in hibernation.

6. Some of other amino acids and related compounds were found in different amounts in the brains of animals of different species.

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A NEW CRITERION OF THE EVOLUTION OF METABOLIC PATHWAYS BASED ON THE THERMODYNAMIC THEORY OF IRREVERSIBLE PROCESSES*

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Various suggestions concerning the criteria of biological evolution or natural selection or the survival of the fittest have been proposed from the viewpoint of thermodynamics by Oparin and the other investigators (*1–6*). In the present paper, the author will propose a new criterion of the evolution of metabolic pathways based on the thermodynamic theory of irreversible processes, and discuss its validity and relations to the other criteria.

I. Some Criteria Which Have Ever Been Proposed

Oparin (*1*) has suggested that the instability and low energy efficiency of fermentation of various types may be considered as a criterion of the evolution of fermentative organisms. But this criterion is not available when we compare two different systems with the same or nearly the same energy efficiencies, *e.g.*, tri- and dicarboxylic acid cycles (see Section III).

The concept of energy efficiency does not contain the factor of " time " which is indispensable for the consideration of " life ". The fact that the classical thermodynamics could not explain the physical aspects of biological processes was also due to its failure to take the factor of " time " into consideration. The " thermodynamics of irreversible processes " is at present the only theory that has succeeded in introducing the conception of " time " into thermodynamics. This new thermodynamic theory may be called a " phenomenology of biological system " because it is valid to represent irreversible processes in an open system of any kinds.

Before the development of this new theory, Lotka (*2*) has already suggested that natural selection tends to make the energy flux through the system a maximum. The " energy flux " has been defined by him as the available energy absorbed by and dissipated within the system per unit time. Rashevsky (*3*) has used the term " energy flux " as the energy exchange between biological units or between a biological unit and its environment. In his definition the quantity to be maximized is the total positive flow of energy. Thus he has given a different meaning from that of Lotka to the maximum principle.

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* This work was printed in Japanese on the " *Seibutsu Kagaku* " (*J. Biol. Sci.*), **8**, 150 (1956).

From the viewpoint of the new thermodynamics, Prigogine and Wiame (*4*) have suggested that the evolution of biological systems is towards the state of minimum entropy production and during this evolution the entropy contained in the system may decrease whilst its heterogeneity increases. They have thus been led to suggest a physico-chemical interpretation of Lamarckism. According to them, Lamarck thought that the evlution of organism seemed to be essentially the consequence of an intrinsic tendency of the living matter toward " complication ". They have considered that the state of minimum entropy production corresponds approximately to the state of minimum metabolism. This standpoint has misled them to the inconsistent conclusions, *i.e.*, the loss of synthetic power in the parasitic bacteria is regarded as the biochemical evolution and the autotrophic organism is more primitive than the heterotrophic one. The reason is that these conclusions are inconsistent with the proposition of Lamarckism but are in harmony with the idea that the evolution is tending towards " simplification ".*

On the other hand, Odum and Pinkerton (*5*) have presented a maximum principle which is also based on the thermodynamic theory of irreversible processes. Their proposition is that natural systems perform at an optimum efficiency for maximum power output, which is always less than the maximum efficiency, and that under the appropriate conditions, maximum power output is the criterion for the survival of many kinds of systems, both living and non-living.

Recently Sugita (*6*) has described that the whole history of the organism may have proceeded so as to enlarge the consumption rate of the free energy. This maximum principle has also been derived from the viewpoint of the new thermodynamics.

II. A New Criterion

Prigogine (*7*) has shown that open systems can evolve to the state of minimum entropy production, that is to say, the stationary state. The stationary state appears only under the appropriate conditions imposed by the environment and is stabilized against temporary perturbations from the generalized LeChatelier-Braun's principle. On the other hand, against the changes of the conditions imposed, the system presents the following responses: if the mechanisms of the system can not adapt to the new conditions, a non-stationary state appears, but if the mechanisms are able to adapt, the system shifts to another stationary state. Such a phenomenon may be for example the control of the stationary metabolic flow of the tricarboxylic acid cycle by the Wood-Werkman reaction, which will be discussed in a succeeding paper by the author (*8*).

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* Of course, we often have a case in which the synthetic power of some biological substances, *e.g.*, of some amino acids has been lost in the course of " biological " evolution. It may, however, be doubtful whether the loss of synthetic power is essentially related with the " biochemical " evolution. From the viewpoint of the doctrine of Lamarckism, the loss of synthetic power can not be regarded as one feature of " biochemical " evolution; on the contrary, it should be considered as a sort of degeneration. It may be desirable that the meanings of such biochemical degeneration as the loss of synthetic power in the mechanism of " biological " evolution are searched from the other different viewpoint (see Bernal, J. D., The Physical Basis of Life, Routledge and Kegan Paul Ltd., London, (1951) p. 77).

It may be considered as one feature of the evolution of organism that organisms change the conditions of environment by their biological activities and then transform themselves by adaptation to the resultant new conditions. This is physically homologous to a phenomenon of transition between two stationary states of an open system stated above. It is therefore natural to study the process of biological evolution by comparing these stationary states. The state of minimum entropy production is equivalent to the state of least energy dissipation* provided that the temperature is kept constant. If it is taken into account that biological activities reveal themselves at a constant temperature and are sensitive to heat, we may say from the viewpoint of fitness or "Zweckmässigkeit" that the system which dissipates the available energy at a less rate than the other is more fitting or "zweckmässiger". In the discussion of the primitiveness of metabolizing systems, we have to take into consideration the various factors that affect the evolution, and let us propose the "energy dissipation rate" as a criterion of biological evolution based upon the thermodynamic theory of irreversible processes. If the adaptative evolution of metabolizing systems proceeds toward the fittest direction, we can regard the metabolizing system which dissipates the available energy at a higher rate than the other as the more primitive system.

In another paper (*9*), an approximate equation of the rate of energy dissipation due to the stationary or cyclic process of metabolism is given by the author as follows:

$$T\sigma=(\Delta G-W)/\tau, \qquad (1)^{**}$$

where T is a constant temperature, σ entropy production per unit time, consequently $T\sigma$ energy dissipation per unit time, ΔG the difference of Gibbs' free energy between the initial reactants and the final products of the process, W the work done by the metabolizing system and the time τ is so small that the state variables are linearly related with time.

Let us examine the conditions for a less rate of energy dissipation from equation (1).

(a) In the case of the same τ, $\Delta G-W$ must be less for the less $T\sigma$. When $\Delta G-W$ is small the energy efficiency $W/\Delta G$ is high. In this case, therefore, the present criterion reduces to that of Oparin. Various types of fermentation may have the same or nearly the same value of τ, because they seem to have mostly a common path except terminal steps. We may therefore use the energy efficiency as a criterion for judgement of the primitiveness of some fermentation-types.

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* It means the state where the available energy is dissipated at the least rate.

** The notation ΔG is used in this equation (1) instead of $\Sigma_m G_m$ in equations. (44) and (4) of the paper referred as (*9*).

(b) $\Delta G/\tau$ and W/τ are powers input and output, respectively. In the case of the same power input, the power output must be larger for the less $T\sigma$, and the Odùm-Pinkerton's criterion is included in the author's proposition. Examples of this case were shown in the paper of Odum and Pinkerton (*5*).

(c) In the case of the same $\Delta G-W$, τ must be larger for the less $T\sigma$. When the reaction of multistep cyclic process proceeds in a stationary state, the period τ of one revolution of the cycle is given as the sum of lifetimes τ_i's of cycle components (*9*). If τ_i-values are in the same order, the number of reaction steps must be larger for the less $T\sigma$. When two different systems of the same overall reaction, *i.e.*, of the same ΔG and W or the same energy efficiency $W/\Delta G$ are compared with each other, we may conclude that the system of roundabout pathway with the more multi-steps than the other is more developed. In other words, it may be said that organisms have a tendency to select the pathway of the least resistance, even if it is roundabout, among the possible pathways between the start and finish. Thus the gain of new reaction steps or the complication of the metabolic pattern may be regarded as one feature of biochemical evolution. Such a viewpoint may explain why there are many complicated cyclic processes among the metabolic pathways. Thus, the conclusion being compatible with the doctrine of Lamarckism that makes mention of an "intrinsic tendency to complication" can be derived from the author's criterion. While the author thinks, as stated in the previous section, that such conclusion can not be obtained from the idea of "evolution by metabolic economy", which has been drawn, by Prigogine and Wiame, from the criterion being identical with the author's one under a certain biological condition, namely, at a constant temperature. The primitiveness of the respiratory cycles is a good example of this case (c) and it will be discussed in the following section.

III. Application to Respiratory Cycles

It is known that the tricarboxylic acid (TCA) cycle exists in an extensive range from higher animals and plants to yeast and bacteria. The TCA cycle, however, does not necessarily play an important role in the terminal steps of respiration. Some microorganisms seem to have other mechanisms of terminal steps. For example, it is believed that the terminal step in *E. coli* or acetate-grown *Aerobacter aerogenes* is the dicarboxylic acid (DCA) cycle (*10*).

In the complete oxidation of one molecule of acetic acid, the TCA cycle yields eleven molecules of ATP through the consecutive reactions of eleven steps. While in the DCA cycle, the detailed mechanism of the first step, *i.e.*, of dehydrogenative condensation of acetic acids is unknown so that there is nothing for it but to assume. The values of phosphorylation quotients, P/O, for various metabolic processes are generally in the range of 2~4. It may, therefore, be assumed that 2~4 molecules of ATP are generated in the reaction of condensation. Thus the DCA cycle probably yields 10~12 molecules of ATP in the complete oxidation of one molecule of acetic acid. Both cycles liberate the same amount of Gibbs' free energy ΔG in the complete oxidation of acetic acid. If the works W done by both cycles are compared with each other in regard to the number of molecules of ATP generated, both cycles may have the same or nearly the same efficiency $W/\Delta G$. This belongs in the case of (c) in the previous section. The periods of both cycles are unknown but the number of reaction steps in the DCA cycle seems not to be more than that in the TCA cycle. It may therefore be concluded for the present that the DCA cycle is probably more primitive than the TCA cycle.

This conclusion is based upon the assumption mentioned above and the thermodynamical considerations, and it goes without saying that it should be reconsidered by the introducing of the proper conditions obtained experimentally and of other factors than thermodynamical.

IV. Conclusions

From the viewpoint of the thermodynamics of irreversible processes, the author porposes to use an approximate equation (1) for the dissipation rate of the available energy of a metabolizing system as a criterion of the evolution of metabolic pathways. It includes as special cases the criteria presented by Oparin and by Odum and Pinkerton. It suggests a solution of the problem of primitiveness of the respiratory (TCA and DCA) cycles, which can not be resolved by the Oparin's criterion. The author's suggestion is that organisms probably select the pathway of the least resistance among the possible ones and then gain new reaction steps and complicate the metabolic patterns. It is compatible with the doctrine of Lamarckism which speaks about an " intrinsic tendency to complication " and is contrary to the idea of "evolution by metabolic economy" introduced by Prigogine and Wiame.

SUMMARY

The author surveys the various suggestions presented from the thermodynamical viewpoint concerning the criteria of biological evolution (Section I).

As a thermodynamical criterion for the judgement of the primitiveness of metabolic pathways, an approximate equation for the dissipation rate $T\sigma$ of the available energy of the metabolizing system:

$$T\sigma=(\Delta G-W)/\tau$$

is proposed, where ΔG is the difference of Gibbs' free energy between the initial reactants and the final products of the metabolic process, W the work

done by the system and τ is the time required for the one process. From this criterion, that of Oparin and that of Odum and Pinkerton are derived as two special cases, respectively. The third special case leads us to a suggestion that organisms have probably proceeded towards the direction of a longer period of the metabolic process, from which the gain of new reaction steps and the complication of the metabolic patterns are derived (Section II).

The primitiveness of the tri- and dicarboxylic acid cycles, which can not be inferred from the Oparin's criterion, is discussed as a good example of the last case (Section III).

ADDENDUM

The author's criterion is a kind of minimum principle. As stated above, this minimum principle leads to a suggestion that organisms perhaps tend to select a pathway of the least resistance among some possible ones, even though it is more roundabout than the others. This suggestion can also be obtained from the maximum principle proposed by Prof. Sugita (*6*). It may therefore be expected that the two principles mentioned above are different expressions, obtained by different variations, of the same fact. Prof. Sugita has referred to the relations of these two principles in his recently published paper on the maximum and minimum principle of the streaming system which includes the living body (*11*).

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